MOLECULAR ASSAYS FOR THE DETECTION OF INVASIVE TUNICATES AND PHYLOGEOGRAPHY OF A TUNICATE INVASION IN PRINCE EDWARD ISLAND

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Abstract

There are currently four invasive tunicate species which are causing significant challenges to the aquaculture industry in Atlantic Canada: Ciona intestinalis, Styela clava, Botryllus schlosseri and Botrylloides violaceus. These tunicates foul mussel socks, aquaculture gear, boat hulls and wharves. As with many aquatic invasive species, detection of these tunicates mostly occurs once adult populations have reached significant population sizes. However, once populations have reached these levels, there is often little that can be done to stop or slow the invasion. Having efficient and sensitive assays that could detect invasive tunicates at microscopic stages would be advantageous as management strategies could be implemented before populations spike to invasive levels. Such an assay would also be useful in monitoring mussel processing plant effluent so that invasive tunicate eggs and larvae are not spread through effluent water discharge to adjacent bays. Molecular assays have been developed in this study that can detect the four invasive tunicate species in both mussel processing effluent water and in bay water surrounding mussel leases. These assays are highly specific and have a sensitivity of detection of 1-5 eggs and/or larvae per water sample. In addition qRT-PCR assays have been developed that can detect and distinguish between different life stages of Ciona intestinalis (egg and larvae) in water samples. This qRT-PCR assay also has the capacity to evaluate viability of free swimming larvae so that nonviable larvae do not cause false positives during mussel processing plant effluent monitoring. The high throughput capacity, high specificity and sensitivity of these assays shows excellent potential for use as a monitoring tool for aquatic invasive species in screening ballast water, effluent waste from shellfish processing plants, as well in local bays and rivers. This study also used phylogenetic analyses of the cytochrome oxidase 1 gene to determine that populations of Botryllus schlosseri in Prince Edward Island have low genetic diversity. Only two haplotypes of B. schlosseri were found in this study in PEI, one which was found in all aquaculture bays tested and the other found only on native substrate in one bay. It is likely that local activity spread this species to other areas of Prince Edward Island after the initial invasion. Phylogeographic analysis suggests that this species was likely transported from Massachusetts to Nova Scotia and then was transported to Prince Edward Island via local boating activity or through the movement of aquaculture species. Through the development of these efficient detection methods and by determining source populations and possible vectors responsible for transporting invasive species to this region, it is hoped that new invasions of aquatic invasive species can be prevented and/or managed before they pose a risk to the aquaculture industry in Prince Edward Island.

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Chapter 1: General Introduction

1.1 Overview of the Mussel Aquaculture Industry in Prince Edward Island

The mussel aquaculture industry is a significant component of the economy of Prince Edward Island contributing $36.3 million of the provincial gross domestic product (GDP) and reaching sales of over $107 million in 2004. Locally, this industry supports over 622 full-time jobs, mostly in rural areas of PEI where employment opportunities are often limited. The industry consists of 123 growers, 7 mussel processing plants and over 10 831 acres of mussel leases (DFO 2006; PEIAA 2009). The Prince Edward Island mussel aquaculture industry also plays a significant role internationally, as PEI grows 80% of the blue mussels produced in Canada and 71% of the mussels grown in North America (DFO 2006; PEIAA 2009).

In PEI, mussels are grown on long-line suspension systems with mussel socks hanging vertically in the water column at an average interval of 44 centimetres (Drapeau et al. 2006). To obtain mussel seed for the grow-out phase collector lines are suspended in the spring to facilitate the settlement of mussel larvae which are naturally present in the water at this time. These seed mussels are allowed to grow to 1- 2.5 centimetres on the collectors before being harvested in the fall (less typically in the spring) and placed into grow-out mussel socks at a density of 120-240 mussels per 30 centimetres (DFO 2006 PEIAA 2009). Mussels remain in the socks for 18-24 months until market size is reached (5.5-6 centimetres) (Drapeau et al. 2006; DFO 2006; PEIAA 2009). After being harvested, mussels are transported to one of seven mussel processing plants where they are removed from socks, declumped, cleaned, graded and have byssal threads removed (DFO 2006).

Mussel processing plants use both mechanical equipment and high pressure water to clean, declump and remove byssal threads (DFO 2006).

1.2 Overview of Invasive Tunicates in Prince Edward Island

Between 1998 and 2004, four invasive tunicate species became established in Prince Edward Island: Ciona intestinalis (Vase Tunicate), Styela clava (Club Tunicate), Botryllus schlosseri (Golden Star Tunicate) and Botrylloides violaceus (Violet Tunicate) (Ramsay et al. 2008).

1.2.1. Styela clava

The club tunicate is a native species in the Pacific Ocean with a native range extending across Siberia, Japan, Korea, northern China, and Shanghai (Cohen 2005). This tunicate has spread beyond its native range to many locations including the Atlantic and Pacific coasts of the United States, Europe, Mediterranean, Australia and Asia (Minchin & Duggan 1988; Lambert & Lambert 1998; Clarke & Therriault 2007; Davis & Davis 2007; Davis & Davis 2008) The club tunicate was the first invasive tunicate to become established in PEI and was first detected in 1998 in the Brudenell River (DFO 2006). Since 1998, the club tunicate has spread on PEI from the Brudenell River to Cardigan River, Montague River, St. Mary's Bay, Orwell River, Vernon River, Murray River, March Water and Darnley Basin, Southwest River, New London, Trout River, and Malpeque Bay (DFO 2006; Gill et al. 2008). It is believed that this spread across PEI was facilitated through transfer of mussel spat and aquaculture gear, as well from processing mussels from affected bays in processing plants located in unaffected bays and recreational boating (Locke et al. 2009).

S. clava is a solitary, hermaphroditic tunicate capable of spawning at 15^{o}C and above from June through October in Prince Edward Island (Bourque 2006). Gametes are shed into the water column and fertilization occurs externally. Larvae are generally present in the water column for 24 hours before settlement occurs. S. clava larvae settle on hard substrate including rocks, shells, wharf pilings, buoys, boat hulls and other natural and artificial substrates. S. clava typically grows up to 12-15cm in length and has a leathery tunic (DFO 2006).

1.2.2 Botryllus schlosseri

The Golden Star tunicate is believed to be native to either the Mediterranean (Berrill 1950) or the Pacific Ocean (Carlton 2005). However, this colonial tunicate has now spread to every continent except Antarctica (Van Name 1945; Yund & O'Neil 2000). The Golden Star tunicate was first reported in Prince Edward Island in 2001 in St. Peter's Bay (DFO 2006; Locke et al. 2009). Since this initial detection on PEI, B. schlosseri has become established in 13 other areas of Prince Edward Island including: Savage Harbour, Tracadie Bay, South Lake, Souris Harbour, Boughton River, St. Mary's Bay, Murray River, Orwell River, Charlottetown Harbour, Port Borden, Summerside Harbour, West Point and Alberton Harbour (Art Smith, Aquatic Invasive Species Coordinator, Department of Fisheries and Oceans, Canada, personal communication).

B. schlosseri is a colonial tunicate with zooids arranged in a star-like pattern around a common excurrent siphon (Van Name 1945). There are many colour variations within this species including: purple, green, orange, yellow, white, brown, black and gray (Carver et

al. 2006). Colonies are typically not mono-coloured but consist of coloured zooids with different coloured margins of the colony between the zooids (Van Name 1945). Colonies of B. schlosseri reproduce both sexually and asexually. Sexual reproduction and fertilization occurs internally within the colony and larvae are brooded within the atrial cavity until the tadpole stage is reached. Each zooid typically contains 1-3 ovaries which are located anterior to the testes and each ovary contains 1 developing egg (Van Name 1945). The tadpole larvae are free-swimming for approximately 36 hours before settling on hard substrates (Berrill 1950). Asexual reproduction occurs via buds or blastozooids which develop from mature colonies. As blastozooids develop, mature zooids are resorbed into the colony. Asexual reproduction allows colonies to expand rapidly as colony size doubles every of 2-3 or 8 days depending on water temperature (Grave 1933; Boyd et al. 1986). Colonies can also reproduce by fragmentation where fragments of a colony break apart and resettle in a new area (Bullard et al. 2007).

1.2.3. Ciona intestinalis

C. intestinalis is native to the Northeast Atlantic Ocean but has spread to Australia, Asia, Africa, Mediterranean, South America and the Atlantic and Pacific coasts of North America (Van Name 1945; McDonald 2004; de Oliveira Marins et al. 2009). Mitogenomic studies have now revealed that individuals previously identified as Ciona intestinalis actually group as two different species: Ciona intestinalis A and Ciona intestinalis B (Iannelli et al. 2007). C. intestinalis was first detected in PEI in the Montague River in 2004 (Ramsay et al. 2009). Since 2004, C. intestinalis has spread throughout PEI to Brudenell River, St. Mary's Bay, Murray River, Cardigan River, Boughton River and Charlottetown Harbour.

Ciona intestinalis is a solitary tunicate with a translucent tunic and can grow to a length of 15 centimetres (Van Name 1945; DFO 1006). Ciona intestinalis spawns at water temperature above 8^{o}C which in Prince Edward Island corresponds to continuous spawning from mid June to late November (Ramsay et al. 2009). C. intestinalis has a large reproductive output with multiple generations growing per year in Prince Edward Island (Ramsay et al. 2009). C. intestinalis are broadcast spawners with fertilization occurring externally in this species. Free swimming larvae hatch from fertilized eggs within 17-24 hours and remain motile in the water column for 6 hours to 4 days (depending on water temperature and region) before settling on a hard substrate (Millar 1952; Dybern 1965).

1.2.4. Botrylloides violaceus

Botrylloides violaceus is a colonial tunicate native to the Pacific Northwest (Japan) (Berill 1950). However, this colonial tunicate has spread throughout the world and is now found along the west and east coasts of North America, Europe and Australia (Carver et al. 2006). B. violaceus was first identified in Prince Edward Island in 2004 in Savage Harbour (DFO 2006). Since 2004, this species has spread throughout PEI to Cardigan Bay, Brudenell River, Port Borden, St. Peter's Bay, Rustico Bay and March Water (DFO 2006).

B. violaceus has different mono-coloured morphs ranging from tan, pink, violet and bright orange. Zooids typically have one ovary on each side posterior to the testis and larvae develop in brood pouches that are formed from extensions from the body wall. Larvae hatch from the brood pouches once they reach the free swimming larval stage (Van Name 1945). Colonial tunicates such as B. violaceus can also reproduce via fragmentation when

portions of a colony break away from the main colony, float in the water column and then reattach in a new area. Bullard et al. (2007) found that of four colonial tunicates studied, B. violaceus had the highest success rate in reattachment following colony fragmentation. This is of concern for mussel aquaculture, as colonies are often fragmented during removal in bays and in mussel processing plants.

1.3 Impact of Invasive Tunicates on Prince Edward Island Shellfish Aquaculture

The mussel aquaculture industry on Prince Edward Island has been significantly impacted by the invasive tunicates since their arrival in 1998. Tunicates have been heavily fouling aquaculture gear, buoys, boats, and mussel socks which have increased labour costs associated with the removal of fouling material (Thompson & McNair 2004). Heavy tunicate fouling has also been shown to exceed the strength of the byssal threads produced by the mussels so when socks are lifted out of the water at harvest, the mussels peel off the sock and fall into the water (Gill et al. 2007). To reduce the level of tunicate fouling on mussel socks, growers have been forced to implement routine treatments into their husbandry practices to reduce the level of tunicate fouling on their mussels. These treatments have significantly increased labour and equipment costs for each grower (DFO 2006). Although many treatments have been tested, the most common include treatment with hydrated lime, acetic acid, and pressure treated water sprays (Gill et al. 2007). In bays with multiple tunicates, C. intestinalis has become the most significant problem to the mussel aquaculture industry due to its weight and the rate at which it can outgrow other tunicates on mussel lines (Ramsay et al. 2008).

Invasive tunicates have also had a significant impact on mussel processing plants. New processing equipment needed to be designed to remove tunicates from mussel socks. The tunicate fouling material has also clogged equipment slowing processing time. Processing costs have also increased due to elevated trucking costs associated with transporting waste fouling material from the processing plants. In addition, processing plants have been required to implement waste water system treatments to reduce the risk of tunicate material exiting effluent from plants into adjacent bays (DFO 2006; Gill et al. 2007). Research has shown that millions of tunicate eggs and larvae were being released in mussel processing plant effluent water adding propagules for new invasions into adjacent bays (Bourque 2006). To mitigate this risk, processing plants are currently testing effluent treatment methods to remove this tunicate material from effluent water. It is critical for industry to have efficient methods to monitor effluent content to ensure that mitigation methods are effective.

Federal regulations have restricted the transfer of mussels and mussel spat from infested bodies of water to uninfested bodies of water. This has had significant impact on availability of mussel seed for some growers. In areas with high tunicate fouling, less spat settles per collector due to tunicates fouling the collector lines (Gill et al. 2007). Processing plants have also been forced by government regulations to adjust which mussels they process based on the fouling organism present in the processing plant's bay and the mussel sock's bay (Gill et al. 2007). Extensive educational campaigns have been launched to inform growers, processors, recreational boaters, other fishermen and the general public on the identification of invasive species present on PEI and to report sightings.

In addition to increasing labour costs associated with the removal of tunicates from mussel lines, there has been much speculation on whether tunicate fouling has a significant impact on mussel growth. Daigle & Herbinger (2009) found that in Nova Scotia, mussels heavily fouled with Ciona intestinalis were smaller and had a lower condition index than mussels grown on unfouled mussel socks. This may be attributed to the competition for food particles since tunicates and mussels overlap in the sizes of food particle that they can filter (Daigle & Herbinger 2009). However, in a preliminary study in PEI, Gill et al. (2007) showed the tunicate fouling did not have an impact on mussel growth or condition index.

1.4 Early Detection of Invasive Tunicates

Since it has been shown that invasive tunicates can reach nuisance population levels within the first few years of being introduced to a region (Ramsay et al. 2008; Arsenault et al. 2009; Ramsay et al. 2009), early detection is critical to initiate rapid responses and mitigation strategies (Mehta et al. 2007; Ramsay et al. 2009). Current detection methods for invasive tunicates in PEI include monitoring recruitment levels of tunicates on PVC plates in various bays and rivers and SCUBA dive surveys in areas where a tunicate has been seen by a member of industry or the general public. While effective at detecting tunicates, these two methods generally only detect tunicates when they have grown to a large size and their population has expanded to a level that can be visually seen. Once population levels have reached this size (and usually include individuals that are reproducing) it becomes very challenging to eradicate or control the population.

In Prince Edward Island, the provincial government's Department of Fisheries, Aquaculture and Rural Development (DFARD) monitors water in mussel lease areas on a weekly basis for the presence and quantity of eggs and larvae of invasive tunicates

(DFARD 2009). This information is made available to mussel growers so they can be prepared for the new recruits of tunicates that will grow from the generation of larvae. Provincial biologists use microscopes to scan water samples for the presence of eggs and larvae of each species of tunicate (Gill et al. 2007). This process is extremely time-consuming which limits the number of samples that can be processed at one time. In addition, staff must be highly trained to distinguish between the eggs and larvae of different species of invasive tunicates and native species in the water samples.

Due to their high level of sensitivity, efficacy and specificity DNA based assays are increasingly being developed as early detection assays for the eggs and larvae of aquatic invasive species in surface and ballast water samples since large quantities of water can be screened for trace amounts of larvae in a high throughput manner (Washington State Aquatic Nuisance Species Committee 2005; Patil et al. 2005; Boeger et al. 2007; Darling & Tepolt 2008). These molecular assays are so sensitive that they can often detect as little as 1 egg or larva in a water sample. Molecular assays have now been developed to detect the invasive crabs Carcinus maenas and Carcinus aestuarii (Darling & Tepolt 2008; Jones et al. 2008), golden mussel larvae Limnoperna fortunei (Boeger et al. 2007), northern Pacific sea star, Asterias amurensis (Deagle et al. 2003) dinoflagellate Gymnodinium catenatum (Patil et al. 2005) and an invasive green algae Caulerpa taxifolia (Harvey et al. 2009). Both the 18S ribosomal gene (18S rDNA) and the cytochrome oxidase 1 gene (COI) have been used in many PCR based studies for species identification because they include highly conserved regions at the species level (Wada et al. 1992; Bell & Grassle 1998; Le Roux et al. 1999; Hare et al. 2000; Mo et al. 2002; Stach & Turbeville 2002; Darling & Tepolt 2008; Harvey et al. 2009).

In Prince Edward Island, PCR based assays would be useful to monitor mussel processing plant effluent for tunicate eggs, larvae and colony fragments. Mussel effluent is high in sediment content which makes microscopic analysis very challenging. With PCR based assays, mussel processing plant effluent could be monitored to ensure that effluent mitigation methods are successful in removing tunicate material from exiting the plant. Early detection assays could also be used to monitor bays and rivers around mussel aquaculture leases and in harbours and ports, as well as ballast water on ships.

While DNA based assays would be useful to identify the species of any egg and larva of invasive tunicates in PEI, they would not be able to determine whether the material detected was viable or nonviable. This is due to the fact that DNA remains very stable post-mortem (Bär et al. 1988). This is particularly relevant in the mussel processing plant effluent monitoring, since mitigation treatments are designed to kill the eggs and larvae of tunicates in effluent making any tunicate material entering a bay through mussel processing plants nonviable and therefore of no risk for further spread into the receiving waters. However, since DNA is a stable molecule, it could still be detected by DNA based assays in nonviable organisms leading to false positives with species specific assays.

To develop a viability assay for invasive tunicate eggs or larvae, mRNA based assays could be developed. mRNA transcripts have varying levels of stability depending on the gene, with some mRNA transcripts degrading within minutes of transcription and others remaining stable for up to 24 hours (Tourrière et al. 2002). The stability of an mRNA transcript is often related to the duration that its translated protein is required in the cell.

Proteins that are quickly produced in response to developmental or external cues are often from mRNA transcripts with short half-lives whereas proteins that are present in stable concentrations over a long time period are generally from mRNA transcripts with long half-lives (Guhaniyogi & Brewer 2001). For gene regulation purposes, mRNA transcripts are often degraded by endonuclease and exonuclease activity (Tourrière et al. 2002). In addition post-death, mRNA is rapidly degraded by RNases (Fontanesi et al. 2008). This characteristic may allow for some mRNA transcripts with short post-mortem half-lives to be used as viability assays in Ciona intestinalis larvae. The degradation of mRNA transcripts post-mortem has been the focus of many recent studies. These studies have shown that mRNA degradation post-mortem is gene dependent, with some mRNA transcripts degrading within minutes of death, and others stable after 48 hours (Zhao et al. 2006; Fontanesi et al. 2008) and even 96 hours (Yasojima et al. 2001). It would be important for surveillance and monitoring programs, especially in programs screening ballast water or mussel processing plant effluent water, to be able to determine whether the larvae being detected in water samples are viable or nonviable organisms. This could be determined by targeting mRNA transcripts that are quickly degraded post-mortem.

Due to the fact that mRNA expression varies depending on the life stage of the organism (Azumi et al. 2007), mRNA based assays could also be developed to distinguish between egg and larval life stages of invasive tunicates in Prince Edward Island. This is useful for monitoring both mussel processing plant effluent and bay water surrounding mussel leases. Such an assay could determine whether mitigation treatments in mussel processing plants were more efficient in killing one life stage over another. In mussel lease areas, growers are interested in knowing when tunicates are successfully reproducing more so than when

they are producing gametes. A life stage specific assay could help distinguish between those two events so that growers know when free swimming larvae are present in water surrounding their mussel socks.

By using a combination of DNA based PCR and RNA based qRT-PCR assays, monitoring for invasive tunicates in Prince Edward Island could increase the ability of both growers and processors to limit the impact and further spread of these four invasive species in this region.

1.5 Molecular Phylogeography of Invasive Tunicates in Prince Edward Island

As species are transported from native populations to new regions throughout the world, several principles have emerged which allow invasions to be tracked from invaded regions back to source populations based on genetic variation of different populations of the same species. Newly introduced populations typically have lower genetic diversity than native populations (Sakai et al. 2001) and newly introduced populations typically share similar genetic haplotypes to the source population (Sakai et al. 2001). This may not be the case if multiple invasions of a species are occurring in the same region. However, in many cases, molecular gene markers can be used to determine temporal and spatial relationships to other populations of that same species (Sakai et al. 2001).

Mitochondrial DNA (mtDNA) is typically used in phylogeographic studies since these genes have a higher rate of mutation over nuclear genes allowing for more resolution below the species level (Hickerson et al. 2010). Mitochondrial DNA is also free of recombination events and has a higher level of neutrality than nuclear DNA (Hickerson et

al. 2010). Nuclear genes have typically been used for resolving higher phylogenetic relationships at or above the species level (Wada et al 1992; Wada 1998; Swalla et al. 2000; Stach & Turbeville 2002; Yokobori et al. 2006; Zeng et al. 2006). However, these characteristic rates of mutation between nuclear and mitochondrial genes are not always true for every species. Many studies now recommend using both a mitochondrial and nuclear marker in phylogenetic studies instead of relying on one gene marker (Pérez-Portela et al. 2009; Hickerson et al. 2010).

Phylogeographic studies have been useful in locating the geographic source of an invasion which also helps elucidate the vector that transported the organism to the new introduced region (Jousson et al. 2000; Sakai et al. 2001). By identifying the vector and source population of an invasion allows researchers to mitigate the risk of future invasions of other species from that same vector and source. In Prince Edward Island, the source populations for the four invasive tunicates are currently unknown. In addition, it is not known whether multiple invasions have spread these invasive tunicates to new areas in PEI or whether the additional yearly expansion is due to local movements from the initial invasions. Finding the source populations for the invasive tunicates on PEI and learning about their spread throughout the region will help managers design effective policies to control further spread of existing invasive species and to protect against new invasions.

1.6 Objectives of this Study

The first objective of this study is to create species specific DNA based assays to detect C. intestinalis, S. clava, B. violaceus and B. schlosseri in water samples. The second objective is to validate the assays in two types of water samples (mussel processing effluent and bay

water surrounding mussel leases) as well as to evaluate the sensitivity and specificity of the assay with two different extraction methods. The third objective of this study is to develop an assay to distinguish between different life stages of Ciona intestinalis in water samples and an assay to evaluate whether larvae detected in water samples are viable or nonviable. The fourth objective is to evaluate the genetic diversity of the populations of B. schlosseri across PEI to determine whether this tunicate was transported to PEI in one invasion or through multiple invasions. In addition, phylogenetic analysis will be conducted with 18S rDNA and COI genes to determine possible source populations for the PEI invasion. It is hoped that these molecular techniques will be able to assist the mussel aquaculture industry in dealing with the challenges associated with invasive tunicates.

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Chapter 2: Development and laboratory validation of species specific assays to detect egg, larvae and adult tissue of invasive tunicate species in water samples[1]

2.1 Introduction

Invasive tunicate species are currently causing challenges in the mussel aquaculture industry in Prince Edward Island, Canada by fouling aquaculture gear, mussel lines, buoys, boat hulls, piers, and other artificial substrates (Thompson & McNair 2004; Ramsay et al. 2008). Between 1998 and 2004, four invasive tunicate species have been detected on PEI including: Styela clava (Club Tunicate), Ciona intestinalis (Vase Tunicate), Botryllus schlosseri (Golden Star Tunicate) and Botrylloides violaceus (Violet Tunicate) (DFO 2006). A native tunicate, Molgula sp. (Sea grape), is also present in PEI but has had less of an impact on aquaculture in this region (Lutz-Collins et al. 2009). Didemnum vexillum is currently not present in Prince Edward Island, but has caused significant fouling problems elsewhere and is currently located on George's Bank, just south of the eastern Canadian/US border (Mercer et al. 2009).

Current monitoring techniques for tunicate species in Prince Edward Island involve the use of recruitment plates which are placed in bays and estuaries, rapid assessments including beach and dive surveys of anecdotal areas of infestation, and a stewardship program focusing on public awareness. Juvenile and adult tunicates obtained by these methods are then identified and, if possible, quantified. However, detecting early developmental stages of these tunicates is more challenging. Water samples are collected and examined under a dissecting microscope to scan for the presence of tunicate eggs and larvae. However, this is

[1] Stewart-Clark SE, Siah A, Greenwood SJ, Davidson J & F Berthe (2009) Development of 18S rDNA and COI gene primers for the identification of invasive tunicate species in water samples. Aquatic Invasions 4: 575-580.

extremely time consuming and requires expertise to visually identify tunicate species at the egg and larval stages. Bourque (2006) showed that millions of tunicate eggs and larvae were being released from mussel processing facilities via effluent outflow into adjacent waters. Current mitigation procedures are being evaluated to determine efficient mechanisms to reduce the load of tunicate propagules in processor outflow. It is clear that high throughput methods need to be developed to efficiently and accurately identify tunicate species at the egg and larval stages.

The objective of this study was to develop sets of specific, unique, and sensitive DNA primers to facilitate the rapid analysis of water samples for invasive tunicate detection by polymerase chain reaction (PCR). Primers specific to the tunicate species mentioned above were developed from published small subunit ribosomal DNA (18S rDNA) and cytochrome oxidase 1 (COI) gene sequences. Both the 18S rDNA gene and the COI gene have been used in many studies for species identification because they include highly conserved regions at the species level (Wada et al. 1992; Bell & Grassle 1998; Le Roux et al. 1999; Hare et al. 2000; Mo et al. 2002; Stach & Turbeville 2002). Primers were first tested for efficacy using samples from as many locations as possible for each species. The specificity of primer sets was then evaluated against the other tunicate species currently present in the area. Lastly, the primer sets were tested for sensitivity using set quantities of egg and free swimming larval life stages present in water samples. Through the development of efficient molecular detection methods and careful monitoring, it is hoped that further invasions of these tunicate species throughout Prince Edward Island waters can be prevented or managed in their earliest stage.

2.2 Materials and Methods

2.2.1 Primer Design

The 18S rDNA sequences for Styela clava (GenBank accession no. **L12442**), Ciona intestinalis (GenBank accession no.**AB013017**), Botryllus schlosseri (GenBank accession no. **AB211066**), and Botrylloides violaceus (GenBank accession no. **AY903927**) were retrieved from GenBank and aligned using ClustalW (Larkin et al. 2007) The COI sequences for Botryllus schlosseri (GenBank accession no **DQ340224**) and Ciona intestinalis (GenBank accession no **AK116803**) were also retrieved from GenBank and aligned using ClustalW (Larkin et al. 2007). Primers were manually designed from 18S rDNA and COI gene regions that were unique to each tunicate species (Appendices 1 & 2).

To minimize false-positives, all primers were assessed to ensure specificity by NCBI-BLAST (National Centre for Biotechnology Information- Basic Local Alignment Search Tool) (Altschul et al. 1997). Primer suitability was further evaluated using IDT Oligoanalyzer 3.0 (Integrated DNA Technology 2007). Multiple primer sets were developed for some species (Table 2.1) and were screened until only the most sensitive and specific primers sets remained.

Table 2.1 Species-specific primer sets designed in this study.

Primer	Sequence 5'-3'	Location	Annealing Temp
CIONAINTEST18S-F1	GTCGACCCGGCCTTACGTCC	661-680	60°C
CIONAINTEST18S-R1	TATCCCTATCACCACCGGGG	1513-1532	60°C
CIONAINTESTCOI-F1	GTCGTTGTTACTTCTCATGCAT	163-184	50°C
CIONAINTESTCOI-R1	CGGATCAAAGAACGTAGTATTAAA	643-666	50°C
STYCLAV18S-F1	GCAAGGCTGGTCACTGG	60-76	58°C
STYCLAV18S-R1	GACACTCGCTGCTTCACTC	874-892	58°C
BOTVIOLET18S-F1	GCGGTCTTGTGCCGGCGACAAACC	268-291	62°C
BOTVIOLET18S-R1	CAGACCTTTCGGCCCAGG	1493-1513	62°C
BOTSCHLO18S-F1	CGAGTCGAACGAAAAGCG	37-54	55°C
BOTSCHLO18S-R1	GGACCTTTCGGCCTAGGAG	1467-1485	55°C
BOTSCHLO18S-F3	CGAGTCGAACGAAAAGCGAAAC	37-58	58°C
BOTSCHLO18S-R3	GACCGACCGCACCCG	617-631	58°C
BOTSCHLO18S-F4	CGAGAGCGAAGGTGGATAACTGTGGT	99-124	60°C
BOTSCHLO18S-R4	ACCGCACCCGCCCAAGA	610-626	60°C
BOTSCHLOCOI-F1	GATAATTAGGAGGTTTGGTAATTGGTTA	81-108	53°C
BOTSCHLOCOI-R1	GACCAATACAGTTCAACAAAACAAAGAT	405-432	53°C
BOTSCHLOCOI-F2	GCCCCCTGCTTTGTTTTTTC	177-196	53°C

2.2.2 Tissue samples and DNA Extraction

Adult tunicate samples were obtained from geographic locations around PEI, within Canada, the USA, and Europe (Table 2.2). These samples were used for efficacy and specificity studies. DNA extractions were performed on fresh or ethanol fixed tissues using QIAamp DNA MiniKits (Qiagen Inc, Canada) according to manufacturer's instructions. Specificity testing involved testing each primer set in PCR with known positive samples of all four invasive species of tunicate currently present in PEI, in addition to the native Molgula sp. and Didemnum vexillum.

For sensitivity studies involving solitary tunicates, both unfertilized eggs and free swimming larval stages were used. Unfertilized eggs were manually collected from C. intestinalis and S. clava specimens in the lab. These eggs were then placed in microcentrifuge tubes filled with filtered seawater in quantities of 1, 5, 10, 20, 50, and 100. The samples were then centrifuged, water was removed and DNA was extracted from the eggs using the QIAamp DNA MiniKits (Qiagen Inc, Canada). Free swimming larvae were produced in the laboratory by removing egg and sperm samples from C. intestinalis and S. clava. These gametes were then placed in Petri dishes with filtered sea water and left for 15-17 hours. Free swimming larvae were collected by pipette and placed in microcentrifuge tubes in 1, 5, 10, 20, 50, and 100 quantities. DNA extractions were then performed as described above.

Table 2.2 Tunicate species and geographic origin of samples used in this study

Ciona intestinalis	**Styela clava**	**Botryllus schlosseri**	**Botrylloides violaceus**	**Molgula sp.**	**Didemnum vexillum**
Montague River, PEI, Canada (46 10 35 N 62 34 15 W)	Montague River, PEI, Canada (46 10 35 N 62 34 15 W)	Orwell River, PEI, Canada (46 08 56 N 62 53 35 W)	Magdalen Islands, Quebec, Canada (47 24 22 N 61 50 41 W)	Boughton River, PEI, Canada (46 15 54 N 62 27 34 W)	Jervis Inlet, BC, Canada (49 50 27N 124 4 56 W)
Woods Hole MA, USA (41 31 25N 70 40 15 W)		Ladysmith, BC, Canada (Orange morph) (49 00 17 N 123 48 57 W)	Woods Hole, MA, USA (41 31 25 N 70 40 15 W)		
Grevelinger Lake, The Netherlands (51 44 21 N 3 49 35 E)		Salem, MA, USA (42 31 12 N 70 52 55 W)	Nanaimo, BC, Canada (Purple morph) (49 12 37 N 123 57 22 W)		
			Salem, MA, USA (Purple and orange morph) (42 31 12 N 70 52 55 W)		

Since the colonial tunicates used in this study spread most often via fragments of adult colonies, individual zooids and small fragments of colonies were used for sensitivity testing for B. schlosseri and B. violaceus. Individual zooids and smaller fragments of zooids were placed in microcentrifuge tubes filled with filtered seawater. The microcentrifuge tubes were then centrifuged, water was pipetted off and DNA extractions were performed using the QIAamp DNA MiniKits (Qiagen Inc, Canada) according to manufacturer's instructions.

2.2.3 PCR

PCR was performed in 25µl final volume containing 12.5 µl AmpliTaq Gold PCR Master Mix (Applied Biosystems, Roche, Branchug, New Jersey), 10 pmol appropriate forward primer, 10 pmol appropriate reverse primer, 25-60 ng appropriate DNA and 9.5 µl sterile ddH_20. Samples were denatured for 3 minutes at 92^oC, amplified over 35 cycles consisting of 1 minute at 94^oC for denaturation, 1 minute at annealing temperatures specific for each primer set [50^oC (C. intestinalis), 53^oC (B. schlosseri), 58^oC (S. clava), or 62^oC (B. violaceus)] for primer annealing, and 3 minutes at 72^oC for elongation. Following the last cycle, polymerization was extended for 5 minutes at 72^oC to complete elongation. PCR negative controls contained PCR Master Mix, primers, sterile ddH_20 and no DNA.

2.2.4 Agarose Gel Electrophoresis

PCR amplicons were separated on 1% agarose gels containing 0.5µg ml^{-1} ethidium bromide. 8µl PCR product and 3µl loading dye were loaded in each lane. 6µl of either 100b or 1kb ladder were loaded in at last one lane of each gel. All agarose gels were run in

TBE Buffer at 135V for 45 minutes. Images were captured under ultraviolet light using AlphaImager 1.0 (Alpha Innotech Corp., San Leandro, CA).

2.2.5 Cloning and Sequencing

PCR amplicons were cloned and sequenced to ensure that the correct target gene was being amplified. The amplicons were inserted into the pCR.2.1-TOPO vector using the Invitrogen TOPO TA cloning kit following the manufacturer's instructions. Plasmids were amplified by transforming One shot® Electrocompetent TOP 10 E. coli cells that were then grown overnight in LB. Plasmids were purified using the Qiagen Plasmid Isolation Miniprep Kit and followed by digestions with EcoRI (Fermentas) and 1% agarose gel electrophoresis to confirm that the plasmid insert was of similar size to the desired PCR amplicon. Plasmid inserts were confirmed by sequencing performed by ACGT Corp. (Toronto, Canada).

2.3 Results

2.3.1 Styela clava

The expected 832 bp amplicon of S. clava DNA was amplified by the STYCLAV18S primer set (Figure 2.1A). This primer set was highly specific and did not amplify DNA of any other tunicate species tested. The primers were able to detect all quantities of both eggs and free swimming larvae tested right down to 1 egg and 1 larva (Figure 2.1B & C). Sequencing confirmed that the amplicon produced was from S. clava 18S based on identity with the 18S rDNA sequence in GenBank (**L12442**).

2.3.2 Ciona intestinalis

The CIONAINTEST18S primer set amplified the expected 871bp fragment of C. intestinalis and did not amplify DNA from any other tunicate species tested (Figure 2.2A). In sensitivity testing this primer set was not able to amplify DNA from neither 1 larva nor 1 egg in water samples. The smallest quantity that was amplified in both replicates in both egg and larval samples was 50, although smaller quantities were detected in some of the replicates (Figure 2.2B).

The CIONAINTESTCOI primer set amplified the expected 503 bp fragment of C. intestinalis COI DNA, but did not amplify DNA from any other tunicate species tested (Figure 2.3A). The CIONAINTESTCOI primer set was able to detect as little as 1 egg and 1 free swimming larva (Figure 2.3B & 2.3C).

2.3.3 Botrylloides violaceus

PCR yielded the expected 1245 bp amplicon when B. violaceus 18S DNA was amplified with the BOTVIOLET18S primer set (Figure 2.4A). Sequencing confirmed that the amplicon produced was from B. violaceus 18S rDNA and was identical to the 18S rDNA sequence in GenBank (AY903927). The BOTVIOLET18S primer set did not amplify DNA from any other tunicate tissue tested. In sensitivity testing, the BOTVIOLET18S primers were able to detect one zooid, and even smaller fragments of one zooid (Figure 2.4B).

2.3.4 Botryllus schlosseri

None of the three primers sets designed to amplify sections of the B. schlosseri 18S gene yielded amplicons following PCR (Data not shown). This result remained consistent despite repeated troubleshooting involving changing annealing temperatures, length of PCR cycles and amount of DNA added to PCR reactions. The primer sets designed to amplify sections of the B. schlosseri COI gene produced the expected amplicon (Figure 2.5A). The BOTSCHLOCOI-F1 and R1 primers did not amplify any DNA from the other tunicates tested in this study. The BOTSCHLOCOIF2 and R1 primer set also successfully amplified B. schlosseri DNA but not those of other tunicate species. The BOTSCHLOCOI-F1 and R1 primers were able to detect as little as a fragment of one zooid present in a water sample (Figure 2.5B).

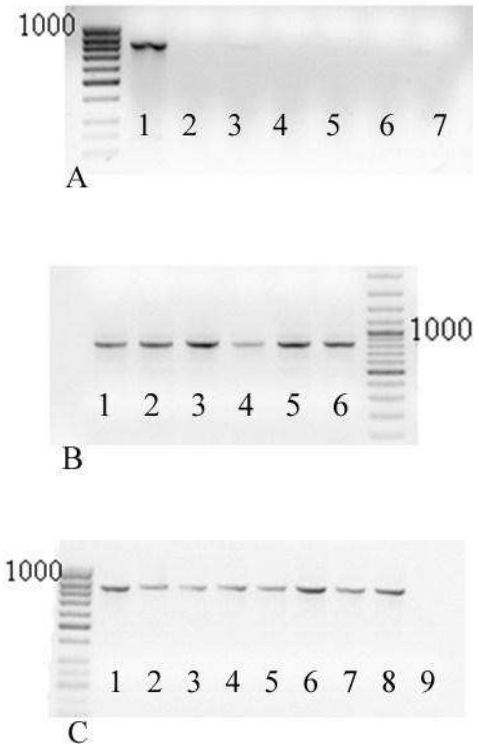


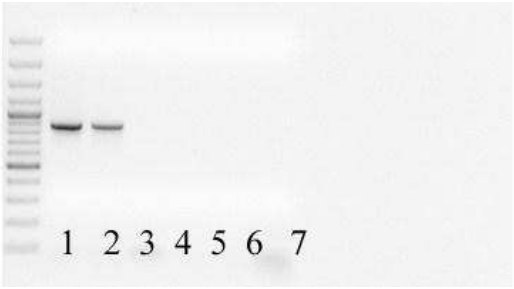
Figure 2.1 Specificity and sensitivity agarose gels of PCR products with STYCLAV18SF1 & R1 primers.

A Specificity agarose gel of PCR products with STYCLAV18S-F1 & R1 primers with: Ln1=S. clava DNA (PEI, Canada). Ln2= C.intestinalis DNA (PEI, Canada). Ln3=B. violaceus DNA (BC, Canada). Ln4=B. schlosseri DNA (PQ, Canada). Ln5=Didemnum vexillum DNA (BC, Canada). Ln6=Molgula sp. (PEI, Canada). Ln7=Negative control.

B Sensitivity agarose gel of PCR products with STYCLAV18S-F1 & R1 primers with: Ln1=1 egg. Ln2=5 eggs. Ln3=10 eggs. Ln4=20 eggs. Ln5=50 eggs. Ln6=100 eggs.

C Sensitivity agarose gel of PCR products with STYCLAV18S-F1 &R1 primers with:Ln1=1 free swimming larva. Ln2=1 free swimming larvae. Ln3=5 free swimming larvae. Ln4=5 free swimming larvae. Ln5=10 free swimming larvae. Ln6=10 free swimming larvae. Ln7=20 free swimming larvae. Ln8=20 free swimming larvae. Ln9=Negative control.

A



B

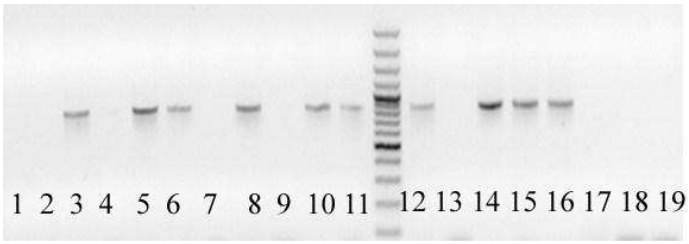


Figure 2.2 Specificity and sensitivity agarose gels of PCR products with CIONAINTEST18SF1 & R1 primer set.

A Specificity agarose gel of PCR products with CIONAINTEST18S-F1 and R1 primer set with: Ln1=C. Intestinalis DNA (PEI, Canada). Ln2=C. Intestinalis DNA (MA, USA). Ln3=S.clava DNA (PEI, Canada). Ln4=B. violaceus DNA (BC, Canada). Ln5=B. schlosseri DNA (PQ, Canada). Ln6=Didemnum vexillum DNA (BC, Canada). Ln7=Negative Control.

B Sensitivity agarose gel of PCR products with CIONAINTEST18S-F1 & R1 primer set with: Ln1=1 free swimming larva. Ln2=5 free swimming larvae. Ln3=10 free swimming larvae. Ln4=20 free swimming larvae. Ln5=50 free swimming larvae. Ln6=100 free swimming larvae. Ln7=1 free swimming larva. Ln8=5 free swimming larvae. Ln9=10 free swimming larvae. Ln10=20 free swimming larvae. Ln11=50 free swimming larvae. Ln12=100 free swimming larvae. Ln13=1 egg. Ln14=10 eggs. Ln15=50 eggs. Ln 16=100 eggs. Ln17=50 eggs. Ln18=1 egg. Ln19=5 eggs.

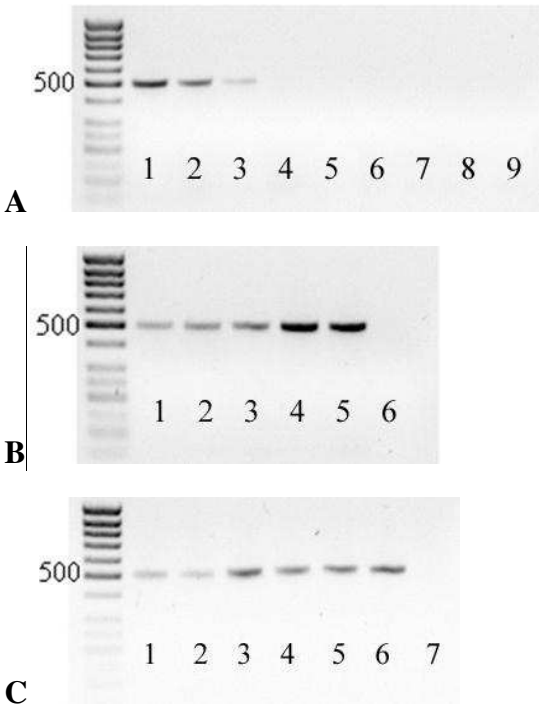
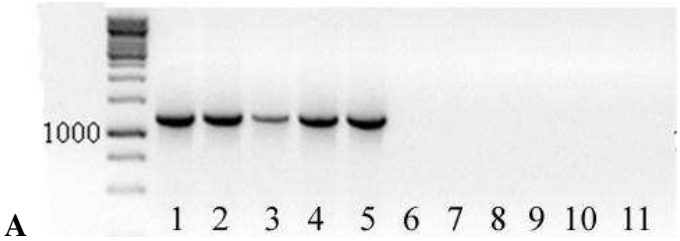


Figure 2.3 Specificity and sensitivity gels of PCR products with CIONAINTESTCOIF1 & R1 primer set.

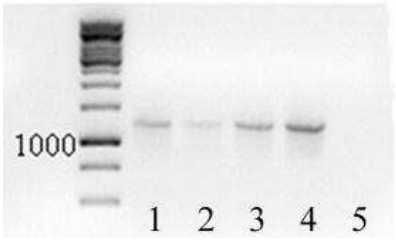
A Specificity agarose gel of PCR products with CIONAINTESTCOI-F1 & R1 primers with: Ln1=C. intestinalis DNA (PEI, Canada). Ln2=C. Intestinalis DNA (MA, USA). Ln3=C.intestinalis DNA (Netherlands). Ln4=S.clava DNA (PEI, Canada). Ln5=B. violaceus DNA (BC, Canada). Ln6=B. schlosseri DNA (PQ, Canada). Ln7=Didemnum vexillum DNA (BC, Canada). Ln8=Molgula sp (PEI, Canada), Ln9=Negative control.

B Sensitivity agarose gel of PCR products with CIONAINTESTCOI-F1 & R1 primers with: Ln1=1 egg. Ln2=5 eggs. Ln3=10 eggs. Ln4=50 eggs. Ln5=100 eggs. Ln6=Negative control.

C Sensitivity agarose gel of PCR products with CIONAINTESTCOI-F1 & R1 primers with: Ln1=1 free swimming larva. Ln2=5 free swimming larvae. Ln3=10 free swimming larvae. Ln4=20 free swimming larvae. Ln5=50 free swimming larvae. Ln6=100 free swimming larvae. Ln7=Negative control.



A



B

Figure 2.4 Specificity and sensitivity agarose gels of PCR products with BOTVIOLETF1 & R1 primer set.

A Specificity agarose gel of PCR products with BOTVIOLET-F1 & R1 primers and: Ln1=B. violaceus (Purple morph) DNA (BC, Canada). Ln2=B. violaceus (Purple morph) DNA (MA, USA). Ln3=B. violaceus (Orange morph) DNA (MA, USA). Ln4=B. violaceus DNA (PEI, Canada). Ln5=B. violaceus (Purple morph) DNA (MA, USA). Ln6=C. intestinalis DNA (PEI, Canada). Ln7=S.clava DNA (PEI, Canada). Ln8=B. schlosseri DNA (PQ, Canada). Ln9=Didemnum vexillum DNA (BC, Canada). Ln10=Molgula sp. DNA (PEI, Canada). Ln11=Negative control.

B Sensitivity agarose gel of PCR products with BOTVIOLET-F1 & R1 primers and: Ln1=1 zooid. Ln2=1 zooid. Ln3=Fragment of zooid. Ln4=Fragment of zooid. Ln5=Negative control.

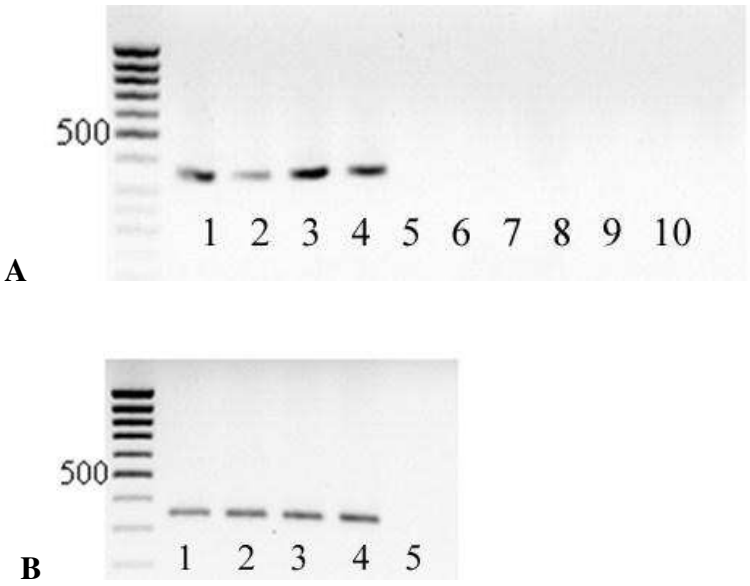


Figure 2.5 Specificity and sensitivity agarose gels of PCR products with BOTSCLOCOIF1 & R1 primer set.

A Specificity agarose gel of PCR products with BOTSCHLOCOI-F1 & R1 primers with: Ln1=B. schlosseri DNA (PEI, Canada). Ln2= B. schlosseri DNA (PQ, Canada). Ln3= B. schlosseri DNA (BC, Canada). Ln4= B. schlosseri DNA (MA, USA). Ln 5=C. intestinalis DNA (PEI, Canada). Ln6=S. clava DNA (PEI, Canada). Ln7=B. violaceus DNA (BC, Canada). Ln 8=Didemnum vexillum DNA (BC, Canada). Ln9=Molgula sp. DNA (PEI, Canada). Ln 10=Negative control.

B Sensitivity agarose gel of PCR products with BOTSCHLOCOI-F1 & R1 primers with: Ln1=1 zooid. Ln2=1 zooid. Ln3=fragment of zooid. Ln4=fragment of zooid. Ln5=Negative control.

2.4 Discussion

Based on the results of specificity and sensitivity testing, primer sets were screened down to one primer set per species (Table 2.3). Our results show that these final primers designed in this study for PCR are suitable for species identification of invasive tunicates at many life stages, including eggs and free swimming larvae. Even when the primers differ by only one base pair and a gap from the target gene sequences of other species, as with the STYCLAV18S primers, they do not cross react in the presence of other tunicate DNA, as tested in this study. This specificity is particularly useful at egg and larval stages where current identification methods between species can be inaccurate and difficult. These primer sets were also able to detect target species when mixed DNA samples from multiple tunicate species were added to PCR reactions (Data not shown). Despite the fact that some tunicate species, such as B. violaceus and B. schlosseri, have different colour morphs, the primers designed in this study are capable of identifying different morphs as one species. This is not surprising since colour is not a taxonomic characteristic used to distinguish between botryllid species (Van Name 1945). In addition, the gene loci involved in colour polymorphism in B. schlosseri have been determined (Sabbadin 1977). The primer sets for all four species in this study were able to detect tunicate DNA from all geographic regions sampled, indicating that the primer regions are not areas of high variability within each species. In addition to the geographic locations sampled in this study, these primer sets have also been successfully used to amplify tunicate DNA from Ireland, Northern Ireland, Spain, and multiple locations within Canada (Stewart-Clark, unpublished data). It is possible that other unsampled geographic locations may have haplotypes with mutations in the

Table 2.3 Final species specific primers laboratory tested to detect invasive tunicates in Prince Edward Island

Primer	Sequence 5'-3'	Size (b)	Annealing Temp
CIONAINTESTCOI-F1	GTCGTTGTTACTTCTCATGCAT	502	50°C
CIONAINTESTCOI-R1	CCGGATCAAAGAACGTAGTATTAAA		
STYCLAV18S-F1	GCAAGGCTGGTCACTGG	832	58°C
STYCLAV18S-R1	GACACTCGCTGCTTCACTC		
BOTVIOLET18S-F1	GCGGTCTTGTGCCGGCGACAAACC	1245	62°C
BOTVIOLET18S-R1	CAGACCTTTCGGCCCAGG		
BOTSCHLOCOI-F1	GATAATTAGGAGGTTTGGTAATTGGTTA	255	53°C
BOTSCHLOCOI-R1	GACCAATACAGTTCAACAAAACAAAGAT		

18S rDNA and COI genes at our primer regions and could go undetected. However this is a limitation of any PCR based assay (Darling & Blum 2007; Hare et al. 2000). It is very important that molecular assays, such as those developed in this study, are validated in their area of use. The presence of PCR inhibitors, or of other species with similar sequences may impact the assay results (Wilson 1997).

The primers designed in this study for solitary tunicates are extremely sensitive as they are able to detect as little as one egg or larva present in a water sample. The primers designed to detect the colonial tunicates are also extremely sensitive as they are able to detect as little as one zooid. While sensitive PCR-based assays have been developed to identify larval forms of other species in water samples such as bivalves (Bell & Grassle 1998; Hare et al. 2000; Wang et al. 2006) no such assays have yet to be developed to detect these invasive tunicates at microscopic stages in water samples.

The species-specific primers generated in this study meet the criteria listed by Darling & Blum (2007) for ideal detection methods for aquatic invasive species: rapid, cost-effective, accurate and accessible. It is hoped that the primer sets designed in this study can become integrated into current monitoring practices for invasive tunicates in Prince Edward Island as well as in other jurisdictions, since the current detection methods of microscopy surveys of water samples is so time consuming and challenging. These primer sets could be used to screen mussel processing plant effluent outflow, mussel growing waters and ballast water for the presence of eggs and larvae of invasive tunicates.

Field trials are the next step in the evaluation process of these assays to ensure that PCR inhibitors, which may be present in water samples from processing facilities, do not limit the efficacy of these primer sets in detecting invasive tunicates (Subject of Chapter 3). Specificity and sensitivity of these primer sets is being evaluated in field water containing various amounts of sediment and organic matter to determine the impact that these compounds have on the diagnostic capacity of these assays. In addition, sampling methods are also being evaluated in field conditions to determine the optimal water sampling techniques and locations within processing facilities to correctly sample for these PCR based assays (Chapter 3).

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Chapter 3: Protocol optimization and field validation of species-specific molecular assays for the detection of tunicate eggs and larvae in mussel processing effluent and bay water

3.1 Introduction

Invasive tunicates are currently causing significant fouling problems to the shellfish aquaculture industry in Prince Edward Island, Canada (Thompson & McNair 2004; Ramsay et al. 2008). To help manage and aid in early detection of these tunicates, species specific PCR based molecular assays have been developed to detect egg and larval stages of four invasive tunicates in water samples (Chapter 2). While laboratory validation occurs under controlled conditions, components of the field sample such as humic acids, phenolic compounds, heavy metals, organic and inorganic chemicals, algae and non-target DNA can inhibit molecular assays and impact sensitivity and/or efficacy (Tebbe & Vahjen 1993; Johnson et al. 1995; Wilson 1997; Toze 1999; Cunningham 2002). Field sampling protocols and sample handling can also limit assay efficacy (Jacobson 1996; Hoorfar et al 2004). For these reasons it is critical for molecular assays and field sampling methods to be validated before assays can be implemented into monitoring and surveillance programs (Cunningham 2002; Jacobson 1996).

According to the Mussel Monitoring System operated by the PEI Department of Fisheries, Aquaculture and Rural Development, and research by the Department of Fisheries and Oceans Canada, two types of water sources on PEI have been highlighted as important areas for monitoring programs for tunicate eggs and larvae. As a result these two different types of field water, bay water surrounding mussel leases and mussel processing plant effluent water, were used in the field validation of the species specifc assays. Many

studies have shown that PCR inhibitors can reduce sensitivity and/or efficacy in molecular assays that involve surface and effluent water samples or other environmental samples that contain sediment (Tebbe & Vahjen 1993; Toze 1999; Xiao et al. 2001; Alexandrino et al. 2004; Kourenti & Karanis 2006; Whitehorse & Hottel 2007). Different methods have been successful in increasing assay sensitivity in the presence of inhibitory compounds, such as the use of specialized DNA extraction protocols or kits that remove PCR inhibitors (Alexandrino et al 2004; Kemp et al. 2006; Behets et al 2007; Whitehouse & Hottel 2007), diluting DNA samples to dilute inhibitory compounds (Verkooyen et al. 1996; Altshuler 2006; Kemp et al. 2006) and altering collection methods to decrease the sample concentration and simultaneously PCR inhibitor concentration (Hoorfar et al. 2004).

In this study, two different methods of water sampling were evaluated for use with these assays: direct grab sampling and concentrated-sieved sampling. Sample type can significantly impact PCR assays since different samples contain different inhibitory compounds and sampling procedures can impact the quantity of target material collected if present (Hoorfar et al. 2004). In addition, two different DNA extraction kits were used to extract DNA from water samples: Qiagen DNeasy Blood and Tissue Kit and Norgen Soil DNA Isolation Kit. Using DNA extraction methods (such as the Norgen kit) with targeted steps to reduce levels of inhibitory compounds in DNA samples have been shown to increase PCR sensitivity in other studies (Alexandrino et al. 2004; Behets et al. 2007; Whitehorse & Hottel 2007). Thirdly, diluting DNA samples by two-fold and ten-fold amounts before PCR was also evaluated as a method to decrease the impact of inhibitory compounds on assay results as has been shown in other studies (Verkooyen et al. 1996; Altshuler 2006; Kemp et al 2006). Through the evaluation of the impact of these three

components of the assay protocol, it is hoped that a standardized assay protocol can be developed and optimized for samples from both field conditions.

Once protocols have been standardized, field validation studies for molecular assays which test sensitivity and repeatability, involve either samples spiked with known quantities of target material (Patil et al 2005; Boeger et al. 2007; Darling & Tepolt 2008), or surveys of environmental samples with unknown quantities of the target species (Jacobson 1996; Boeger et al. 2007). An imperative step in any new assay validation is also a comparison against the current industry standard (Jacobson 1996; Hoorfar et al. 2004; Boeger et al. 2007), which for invasive tunicate monitoring on PEI is microscopic analysis of water samples.

There were three main objectives to this study. 1. To optimize and create standardized assay protocols for samples collected from field conditions 2. To validate assay sensitivity and repeatability with spiked samples in two different types of field water samples and 3. To directly compare assay results from the primers developed in this study with results of the industry standard microscope analysis of bay water samples in PEI.

3.2 Materials and Methods

3.2.1 Water samples

Water samples were collected from two different water types (mussel processing effluent water and bay water) using two different sampling methods (direct grab and sieve concentrated). Mussel processing effluent water was collected from the effluent outflow pipe of Prince Edward Aquafarms mussel processing plant in New London, Prince Edward Island, Canada. Water was collected on July 14, 2008 using both the direct grab and sieve concentrated methods. A 1 litre direct grab sample was collected from the effluent water pipe just before entry into New London Bay and aliquoted into 50ml tubes before freezing at -80^{o}C until further use. The sieve-concentrated samples were collected by passing 30 litres of effluent water flow through a 75µm sieve. The material collected in the sieve was flushed into an Erlenmeyer flask using 1 litre of filtered artificial seawater (Derived from DFO protocol; Daniel Bourque personal communication). The water was then aliquoted into 50 ml tubes and frozen at -80^{o}C until further use. Bay water surrounding a mussel lease was collected in July 2009 from St. Mary's Bay, Prince Edward Island, Canada. A 1 litre direct grab sample was collected from the bay surface water, aliquoted into 50ml tubes and frozen at -80^{o}C until further use. Sieve-concentrated samples were collected by continuously lowering and raising a bilge pump through the water column over a 3 minute period pumping 150 litres of water through a 75µm sieve. Contents collected in the sieve were washed into containers using 500 ml of water (Protocol derived from Gill et al. 2007). Water samples were aliquoted in 50 ml tubes and frozen at -80^{o}C until further use.

3.2.2 Spiking Water Samples

For sensitivity studies involving solitary tunicates, both unfertilized eggs and free swimming larval stages were used. Unfertilized eggs were manually collected from Ciona intestinalis and Styela clava specimens in the lab. These eggs were then placed in microcentrifuge tubes filled with bay or mussel processing effluent water in quantities of 1, 5, 10, 20, 50, and 100. Each quantity was replicated 5 times. The samples were then centrifuged at 14 000 g for 2 minutes, water was removed and DNA was extracted from the pelleted material. Free swimming larvae were produced in the lab by removing egg and sperm samples from Ciona intestinalis and Styela clava. These gametes were then placed in Petri dishes with filtered sea water and left for 15-17 hours to develop into free swimming larvae. Free swimming larvae were collected by pipette and placed in microcentrifuge tubes filled with bay or mussel processing plant effluent water in 1, 5, 10, 20, 50, and 100 quantities. The samples were then centrifuged at 14 000 g for 2 minutes, water was removed and DNA was extracted from pelleted material.

Since the colonial tunicates used in this study spread most often via fragments of adult colonies, individual zooids and small fragments of colonies were used for sensitivity testing for Botryllus schlosseri and Botrylloides violaceus. Individual zooids and colonies were placed in microcentrifuge tubes filled with water samples. Each quantity (zooid and colony) was replicated 5 times for each species. The microcentrifuge tubes were then centrifuged at 14 000 g for 2 minutes, water was pipetted off and DNA extractions were performed. DNA was extracted from bay water using the Qiagen DNeasy Blood and

Tissue kit (Qiagen Inc, Canada) while DNA was extracted from mussel processing effluent water using the Norgen Soil DNA Isolation Kit (Norgen Biotek Corp, Canada).

3.2.3 Optimization of Assay Protocol

DNA extraction protocol evaluation

For each of the four water sample types (mussel processing effluent direct grab, mussel processing effluent sieve concentrated, bay water direct grab, bay water sieve concentrated) two methods of DNA extractions were performed on quantities of 1-100 Ciona eggs or larvae to evaluate the efficacy of each DNA extraction kit in removing PCR inhibitors from the water samples. The two DNA extraction kits evaluated in this study were the Qiagen DNeasy Blood and Tissue Minikit (Qiagen Inc, Canada) and the Norgen Soil DNA Isolation Kit (Norgen Biotek Corp, Canada). For the Qiagen extractions, an extra step was added to the manufacturer's protocol to remove sediment from the DNA sample. Following the addition of Buffer AL, tubes were vortexed and then centrifuged at 14 000 g for 1 minute. The supernatant was transferred to a new 1.5ml tube and 200µl of ethanol was added. The remainder of the protocol followed the manufacturer's directions. The Norgen Kit DNA extractions were all done according to the manufacturer's protocol.

Sample dilution PCR experiment

PCR was performed in 25µl final volume containing 12.5 µl AmpliTaq Gold PCR Master Mix (Applied Biosystems manufactured by Roche, Branchburg, New Jersey), 10 pmol appropriate species forward primer, 10 pmol appropriate species reverse primer, 1-60 ng appropriate DNA and 9.5 µl sterile nuclease free ddH_20. Preliminary PCR tests included DNA samples diluted two-fold and ten-fold to investigate the impact of PCR inhibitors on

amplification. Samples were denatured for 3 minutes at 92^{o}C, amplified over 35 cycles consisting of 1 minute at 94 ^{o}C for denaturation, 1 minute at annealing temperatures specific for each primer set: 50^{o}C (C. intestinalis), 53 ^{o}C (B. schlosseri), 58 ^{o}C (S. clava), or 62 ^{o}C (B. violaceus) for primer annealing, and 3 minutes at 72 ^{o}C for elongation. Following the last cycle, polymerization was extended for 5 minutes at 72^{o}C to complete elongation. PCR amplicons were separated in 1% agarose gels containing 0.5µl/ ml SYBR Green and visualized using ultraviolet light. 20µl of PCR sample and 3 µl loading dye were added to each well. Agarose gels were run in TBE buffer at 165V for 1 hour. 6µl 100b or 1kb ladder were loaded into at least one lane of each gel. Images were captured using VersaDoc Imager (Bio-Rad).

3.2.5 Comparison with Industry Standard

Water samples were collected from seven areas on PEI from August 10-12th 2009 (Table 3.1) as part of the Mussel Monitoring Program by the PEI Department of Fisheries, Aquaculture and Rural Development. Water samples were collected by continuously lowering and raising a bilge pump through the water column over a 3 minute period pumping 150 litres of water through a 64µm sieve. The collected materials were then washed from the sieve into tubes using approximately 25 ml of water. Water samples were brought back to the provincial laboratory where they were spread on a 64µm sieve and then washed into a 100ml beaker with 10ml tap water. 10 ml of water was then removed from the beaker and examined through a microscope with a counting wheel for the presence of tunicate eggs and larvae. Once the samples were surveyed, water samples were returned to collection tubes and maintained at -4^{o}C for 2 days. Samples were then transferred to the molecular lab where they were stored at -80^{o}C until DNA extractions

were performed. This experiment was organized as a blind-trial. The biologist from the PEI Department of Fisheries, Aquaculture and Rural Development (Aaron Ramsay) who conducted the microscope analysis did not know the results of the molecular analysis which was conducted by Sarah Stewart-Clark and vice versa.

For molecular analysis, water samples were thawed and centrifuged at 5250 g for 15 minutes to pellet material. Pelleted material contained so much sediment (>100µl), that Norgen Soil DNA Isolation Kits were used to extract DNA from each sample instead of Qiagen DNeasy Blood and Tissue kits. DNA extractions followed manufacturer's protocol (Norgen Biotek, Guelph Canada). PCR was performed in 25µl final volume containing 12.5 µl AmpliTaq Gold PCR Master Mix (Applied Biosystems manufactured by Roche, Branchburg, New Jersey),10 pmol appropriate species forward primer, 10 pmol appropriate species reverse primer, 1-60 ng appropriate DNA and 9.5 µl sterile nuclease free ddH_20. All DNA samples were diluted ten-fold with nuclease free ddH_2O prior to PCR. Samples were denatured for 3 minutes at 92^oC, amplified over 35 cycles consisting of 1 minute at 94 ^oC for denaturation, 1 minute at annealing temperatures specific for each primer set, 50^oC (C. intestinalis), 53 ^oC (B. schlosseri), 58 ^oC (S. clava), or 62 ^oC (B. violaceus), and 3 minutes at 72 ^oC for elongation. Following the last cycle, polymerization was extended for 5 minutes at 72^oC to complete elongation. PCR amplicons were separated on 1% agarose gels containing 0.1µl/ml SYBR Safe and visualized using ultraviolet light. 20µl PCR product and 3µl loading dye was added to each gel lane. Images were captured using a VersaDoc Imager (Bio-Rad).

Table 3.1 Water samples collected for comparison of molecular assays against current industry standard (microscope analysis)

Date of Water Collections	**Site of Water Collections (PEI, Canada)**
August 10, 2009	Boughton River
August 10, 2009	Cardigan River
August 11, 2009	Montague River
August 11, 2009	St. Mary's Bay
August 11, 2009	Murray River
August 12, 2009	Darnley Basin
August 12, 2009	Marchwater in Malpeque Bay
August 12, 2009	Marchwater
August 12, 2009	Marchwater

3.3 Results

3.3.1 Optimization of Assay Protocols

Bay Water

All DNA samples extracted from various quantities of Ciona larvae in direct grab bay water using the Qiagen DNeasy Blood and Tissue extraction kits were amplified during PCR. Amplicons were generated in all dilution categories including full strength, two-fold and ten-fold dilutions (Figure 3.1A). None of the DNA samples extracted from various quantities of C. intestinalis larvae in direct grab bay water using the Norgen Soil DNA Isolation kit were amplified during PCR. This trend held true in all dilution categories (Figure 3.1B). In sieve-concentrated bay water samples, DNA extracted from all quantities of Ciona eggs using the Norgen Soil DNA Isolation kit were amplified in the ten-fold dilution series. None of the two-fold dilution samples were amplified while the 10-100 larval sample quantities were amplified in the undiluted samples (Figure 3.2A). All DNA samples extracted using the Qiagen DNeasy kit from 1-100 Ciona eggs in sieve-concentrated bay water samples were amplified during PCR in all dilution categories (Figure 3.2B).

Mussel Processing Plant Effluent Water

The only amplicons produced during PCR with mussel processing water samples were extracted using the Norgen Soil DNA Isolation kit. No amplicons were produced in the undiluted or 2X concentrated direct grab Norgen extracted DNA samples however DNA was successfully amplified in two-fold and ten-fold diluted samples (Figure 3.3A). In sieve-concentrated mussel processing water samples, amplicons were produced in the 50

and 100 quantity undiluted samples; however in the two-fold and ten-fold diluted samples, amplicons from as little as 5 eggs were amplified (Figure 3.4A). No DNA samples extracted from C. intestinalis eggs in mussel processing plant effluent using the Qiagen DNeasy kits were amplified during PCR. This trend was seen in both water sample types (direct and sieve-concentrated) and in all DNA sample dilutions used in PCR (1X, 2X, two-fold and ten-fold dilutions) (Figures 3.3B and 3.4B).

3.3.2 Sensitivity Analysis with spiked field water samples

Ciona intestinalis Assay

Bay Water

Ciona intestinalis larvae were detected in 100% of direct grab bay water samples spiked with 1-100 larvae using the CIONAINTESTCOI assay (Figure 3.5A). C. intestinalis eggs were detected in 94% of direct grab bay water samples spiked with known quantities of C. intestinalis eggs using the CIONAINTESTCOI assay (Figure 3.5B). The CIONAINTESTCOI assay successfully detected C. intestinalis larvae in 94% of sieve concentrated bay water samples and in 100% of sieve concentrated water samples spiked with C. intestinalis eggs (Figures 3.5C and 3.5D).

Mussel Processing Plant Effluent Water

C. intestinalis larvae were detected in 100% of direct grab mussel processing effluent water samples spiked with 5-100 larvae but in none of the samples spiked with 1 larva (Figure 3.6A). In sieve-concentrated effluent water samples that were spiked with C. intestinalis larvae, the CIONAINTESTCOI assay detected larvae in 100% of these samples (Figure 3.6B). The CIONAINTESTCOI assay detected C.intestinalis eggs in 93% of direct

grab water effluent water samples spiked with 1-100 quantities of C. intestinalis eggs (Figure 3.6C). This assay also detected C. intesitnalis larvae in 100% of sieve-concentrated effluent water that were spiked with 5-100 larvae but in only 20% of samples spiked with 1 larva (Figure 3.6D).

Styela clava Assay

Bay Water

In direct grab and sieve-concentrated water samples collected from St. Mary's Bay and spiked with known quantities of S. clava larvae, 100% of all quantities tested (1-100) were detected by the STYCLAV18S assay and 97% of all quantities of water samples spiked with S. clava eggs were detected with the STYCLAV18S assay (Figure 3.7A-D).

Mussel Processing Effluent Water

In direct grab mussel processing plant effluent water samples spiked with S. clava larvae, 100% of water samples spiked with 5-100 quantities of larvae were detected with the STYCLAV18S assay (Figure 3.8A). Only 60% of the water samples spiked with 1 larva were detected with this assay. Sieve-concentrated mussel processing plant effluent water spiked with known quantities of S. clava larvae were detected in 100% of samples that were spiked with 5-100 larvae with the STYCLAV18S assay and in only 40% of the samples that were spiked with 1 larva (Figure 3.8B). In direct grab water samples collected from mussel processing plant effluent and spiked with known quantities of S. clava eggs, 100% of all spiked samples were detected with the STYCLAV18S assay (Figure 3.8C). In sieve concentrated mussel processing plant effluent water that was spiked with S. clava

eggs, 100% of samples spiked with all quantities of eggs were detected with the STYCLAV18S assay (Figure 3.8D).

Botryllus schlosseri Assay

Bay Water

The BOTSCHLOCOI assay detected B. schlosseri in 90% of all zooid and colony spiked direct grab bay water samples (Figure 3.9A)

Mussel Processing Plant Effluent Water

The BOTSCHLOCOI assay detected B. schlosseri colonies and zooids in 100% of samples tested from both the direct grab spiked water samples and the sieve-concentrated water samples (Figure 3.9B & C).

Botylloides violaceus Assay

Bay water

Boytrlloides violaceus was detected in 100% of the direct grab bay water samples that were spiked with zooid and colony quantities of B. violaceus using the BOTVIOLET18S assay (Figure 3.10A).

Mussel Processing Plant Effluent Water

The BOTVIOLET18S assay detected B. violaceus in 100% of all direct grab and sieve-concentrated mussel processing effluent water spiked with zooid and colonies of B. violaceus (Figure 3.10B &C).

3.3.3 Comparison with Industry Standard

Using the industry standard microscope analysis, S. clava larvae were detected in water samples from Darnley Basin (58 larvae), Murray River (2 larvae) and Marchwater while C. intestinalis larvae were detected in water samples from Cardigan River (4 larvae), Montague River, Boughton River (48 larvae), St. Mary's Bay (3 larvae) and Murray River (1 larvae). No eggs or larvae from B. violaceus or B. schlosseri were detected in any of the water samples in this study.

Using molecular assays, S. clava was detected in water samples from Darnley Basin and Marchwater while C. intestinalis was detected in water samples from Cardigan River, Montague River, Boughton River, and St. Mary's Bay (Figure 3.11A &B). No material from B. violaceus or B. schlosseri was detected in water samples by the molecular assays (Figure 3.12A &B).

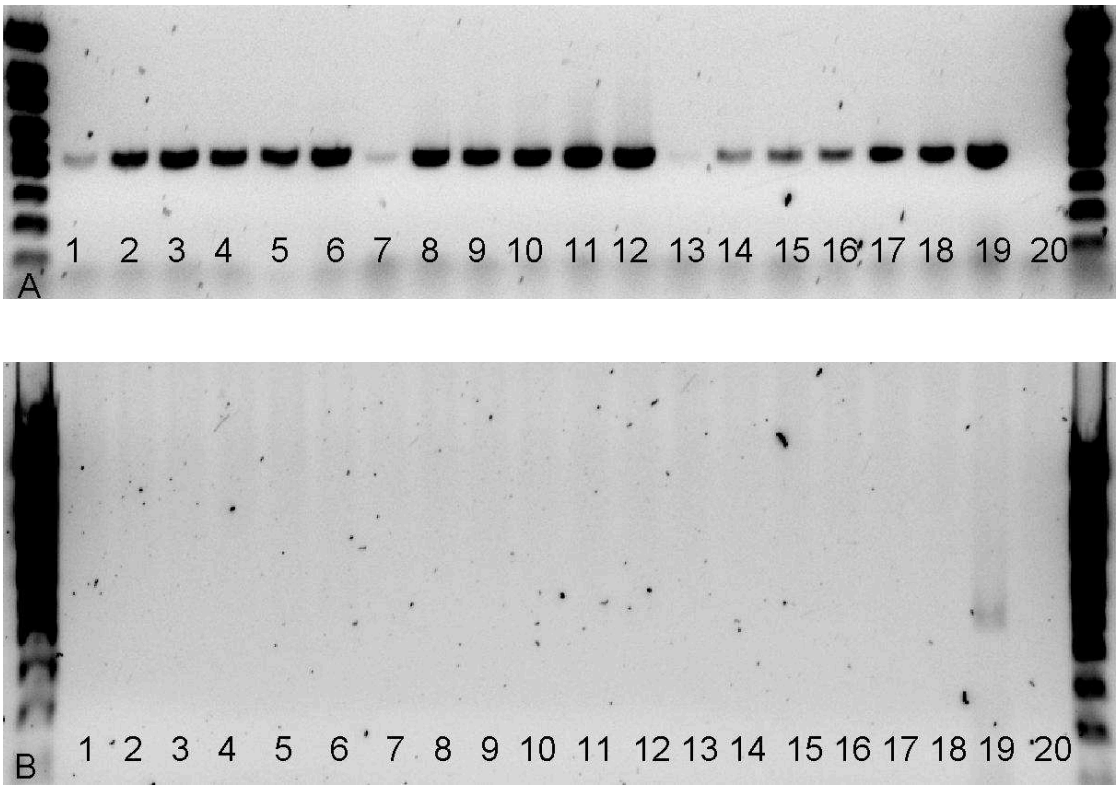

Figure 3.1 A. Agarose gel of PCR amplicons from DNA samples extracted using Qiagen DNeasy DNA extraction kit from direct grab bay water samples spiked with known quantities of Ciona larvae. **B.** Agarose gel of PCR amplicons from DNA samples extracted using Norgen Soil DNA Isolation kit from direct grab bay water samples spiked with C. intestinalis larvae. Ln1=1 larva (undiluted). Ln2=5 larvae (undiluted). Ln3=10 larvae (undiluted). Ln4=20 larvae (undiluted). Ln5=50 larvae (undiluted). Ln6=100 larvae (undiluted). Ln7=1 larva (2-fold dilution). Ln8=5 larvae (2-fold dilution). Ln9=10 larvae (2-fold dilution). Ln10=20 larvae (2-fold dilution). Ln11=50 larvae (2-fold dilution). Ln12=100 larvae (2-fold dilution). Ln13=1 larva (10-fold dilution). Ln14=5 larvae (10-fold dilution). Ln15=10 larvae (10-fold dilution). Ln16=20 larvae (10-fold dilution). Ln17=50 larvae (10-fold dilution). Ln18=100 larvae (10-fold dilution). Ln19= Positive control. Ln20= Negative control.

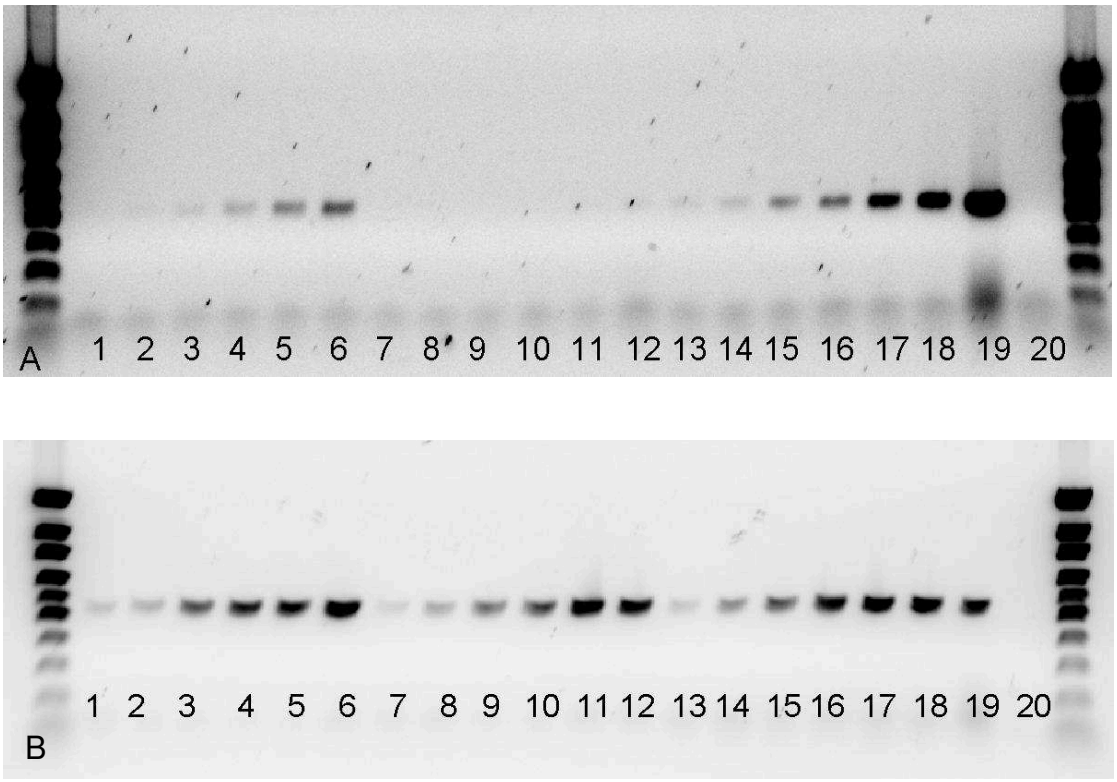

Figure 3.2 A. Agarose gel of PCR amplicons from DNA samples extracted using Norgen Soil DNA Isolation kit from sieve-concentrated bay water samples spiked with known quantities of Ciona eggs. **B.** Agarose gel of PCR amplicons from DNA samples extracted using Qiagen DNeasy kit from sieve-concentrated bay water samples spiked with C. intestinalis eggs. Ln1=1 egg (undiluted). Ln2=5 eggs (undiluted). Ln3=10 eggs (undiluted). Ln4=20 eggs (undiluted). Ln5=50 eggs (undiluted). Ln6=100 eggs (undiluted). Ln7=1 egg (2-fold dilution). Ln8=5 eggs (2-fold dilution). Ln9=10 eggs (2-fold dilution). Ln10=20 eggs (2-fold dilution). Ln11=50 eggs (2-fold dilution). Ln12=100 eggs (2-fold dilution). Ln13=1 egg (10-fold dilution). Ln14=5 eggs (10-fold dilution). Ln15=10 eggs (10-fold dilution). Ln16=20 eggs (10-fold dilution). Ln17=50 eggs (10-fold dilution). Ln18=100 eggs (10-fold dilution). Ln19= Positive control. Ln20= Negative control.

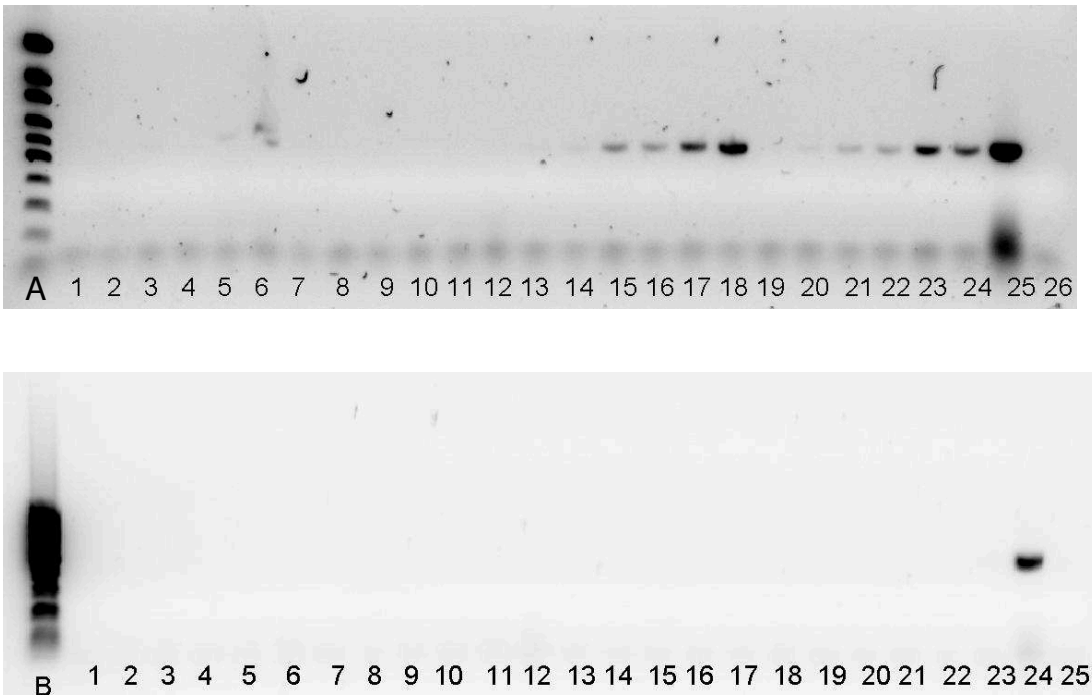

Figure 3.3. A. Agarose gel of PCR amplicons from DNA samples extracted using Norgen Soil DNA Isolation kit from direct grab mussel processing effluent water samples spiked with known quantities of Ciona eggs. **B.** Agarose gel of PCR amplicons from DNA samples extracted using Qiagen DNeasy kit from direct grab mussel processing effluent water samples spiked with C. intestinalis eggs. Ln1=1 egg (undiluted). Ln2=5 eggs (undiluted). Ln3=10 eggs (undiluted). Ln4=20 eggs (undiluted). Ln5=50 eggs (undiluted). Ln6=100 eggs (undiluted). Ln7=1 egg (2X undiluted). Ln8=5 eggs (2X undiluted). Ln9=10 eggs (2X undiluted). Ln10=20 eggs (2X undiluted). Ln11=50 eggs (2X undiluted). Ln12=100 eggs (2X undiluted). Ln13=1 egg (2-fold dilution). Ln14=5 eggs (2-fold dilution). Ln15=10 eggs (2-fold dilution). Ln16=20 eggs (2-fold dilution). Ln17=50 eggs (2-fold dilution). Ln18=100 eggs (2-fold dilution). Ln19=1 egg (10-fold dilution). Ln20=5 eggs (10-fold dilution). Ln21=10 eggs (10-fold dilution). Ln22=20 eggs (10-fold dilution). Ln23=50 eggs (10-fold dilution). Ln24=100 eggs (10-fold dilution). Ln25= Positive control. Ln26= Negative control.

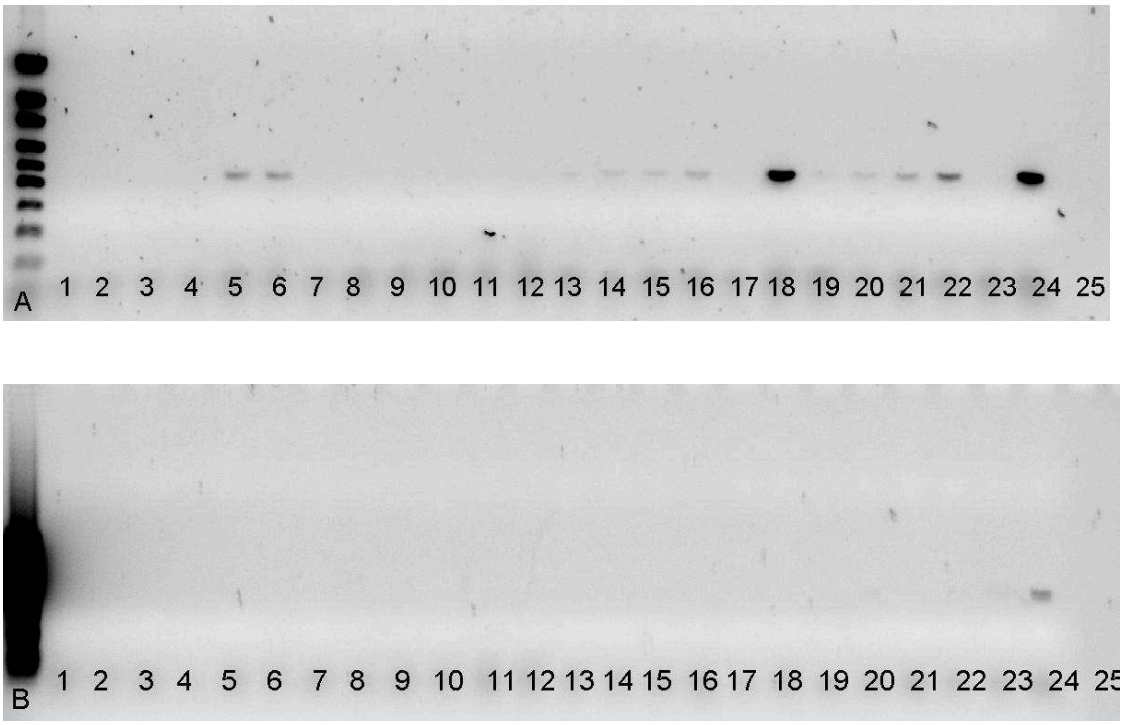

Figure 3.4. A. Agarose gel of PCR amplicons from DNA samples extracted using Norgen Soil DNA Isolation kit from direct grab mussel processing effluent water samples spiked with known quantities of Ciona eggs. **B.** Agarose gel of PCR amplicons from DNA samples extracted using Qiagen DNeasy kit from direct grab mussel processing effluent water samples spiked with C. intestinalis eggs. Ln1=1 egg (undiluted). Ln2=5 eggs (undiluted). Ln3=10 eggs (undiluted). Ln4=20 eggs (undiluted). Ln5=50 eggs (undiluted). Ln6=100 eggs (undiluted). Ln7=1 egg (2X undiluted). Ln8=5 eggs (2X undiluted). Ln9=10 eggs (2X undiluted). Ln10=20 eggs (2X undiluted). Ln11=50 eggs (2X undiluted). Ln12=100 eggs (2X undiluted). Ln13=1 egg (2-fold dilution). Ln14=5 eggs (2-fold dilution). Ln15=10 eggs (2-fold dilution). Ln16=20 eggs (2-fold dilution). Ln17=50 eggs (2-fold dilution). Ln18=100 eggs (2-fold dilution). Ln19=1 egg (10-fold dilution). Ln20=5 eggs (10-fold dilution). Ln21=10 eggs (10-fold dilution). Ln22=20 eggs (10-fold dilution). Ln23=50 eggs (10-fold dilution). Ln24=100 eggs (10-fold dilution). Ln 25= Negative Control.

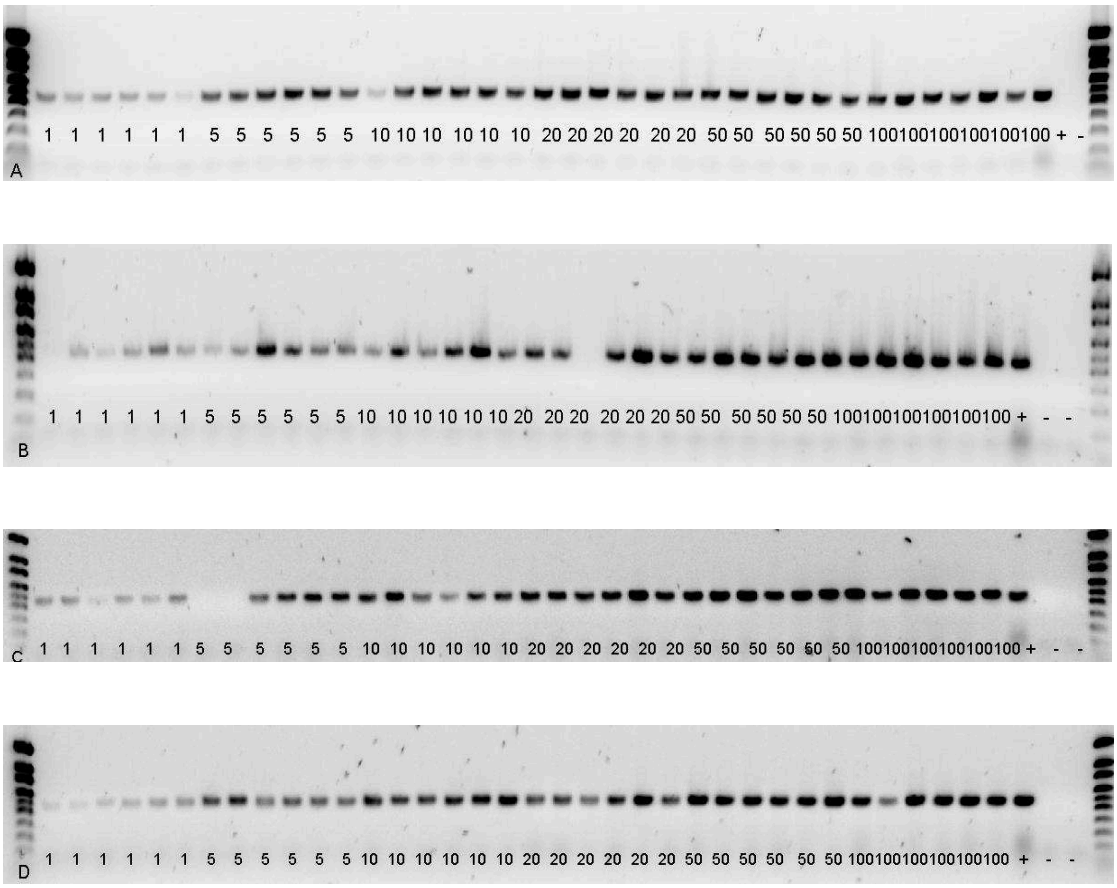

Figure 3.5 Agarose gels of PCR amplicons with CIONAINTESTCOI assay in bay water samples **A.** Direct grab bay water spiked with 1, 5, 10, 20, 50 and 100 C. intestinalis larvae **B.** Sieve-concentrated bay water spiked with 1, 5, 10, 20, 50 and 100 C. intestinalis larvae. **C.** Direct grab water spiked with 1, 5, 10, 20, 50 and 100 C. intestinalis eggs. **D.** Sieve-concentrated bay water spiked with 1, 5, 10, 20, 50 and 100 C. intestinalis eggs.

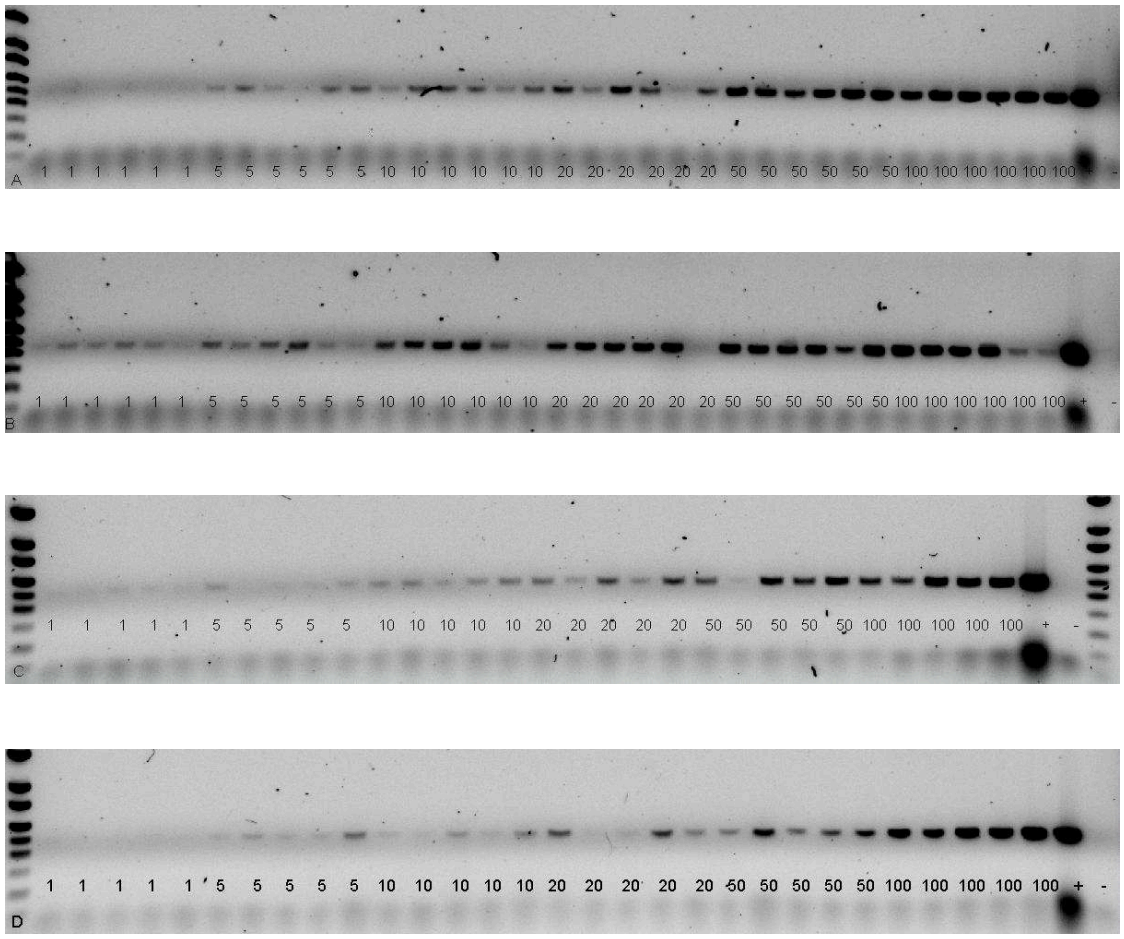

Figure 3.6 Agarose gels of PCR amplicons with CIONAINTESTCOI assay in mussel processing plant effluent water **A**. Direct grab mussel processing plant water spiked with 1, 5, 10, 20, 50 and 100 Ciona intesintalis larvae. **B.** Sieve-concentrated mussel processing plant effluent water spiked with 1, 5, 10, 20, 50 and 100 C. intestinalis larvae. **C.** Direct grab mussel processing effluent water spiked with 1, 5, 10, 20, 50 and 100 C. intestinalis eggs. **D.** Sieve-concentrated mussel processing effluent water spiked with 1, 5, 10, 20, 50 and 100 C. intestinalis eggs.

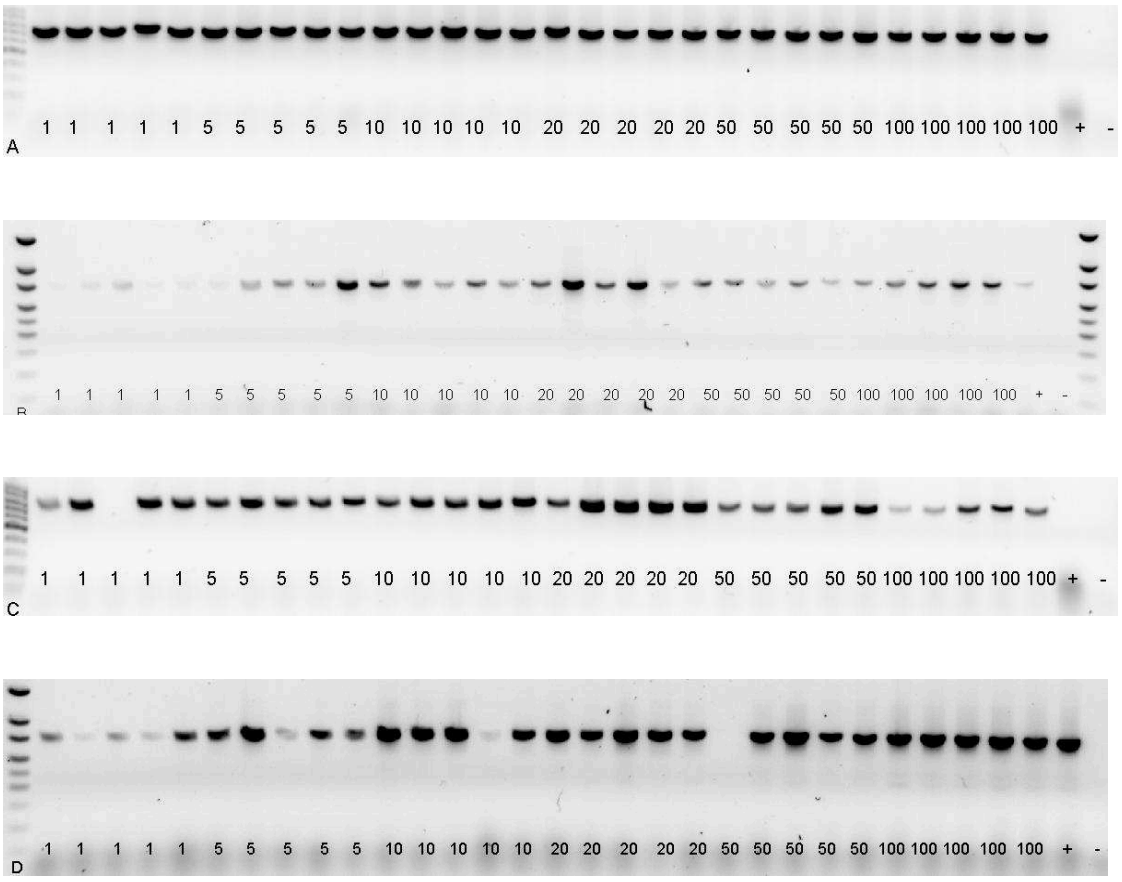

Figure 3.7. Agarose gels of PCR amplicons from STYCLAV18S assay from bay water samples **A.** Direct grab bay water samples spiked with 1, 5, 10, 20, 50 and 100 S. clava larvae. **B.** Sieve-concentrated bay water samples spiked with 1, 5, 10, 20, 50 and 100 S. clava larvae. **C.** Direct grab water samples spiked with 1, 5, 10, 20, 50 and 100 S. clava eggs. **D.** Sieve-concentrated water samples spiked with 1, 5, 10, 20, 50 and 100 S. clava eggs.

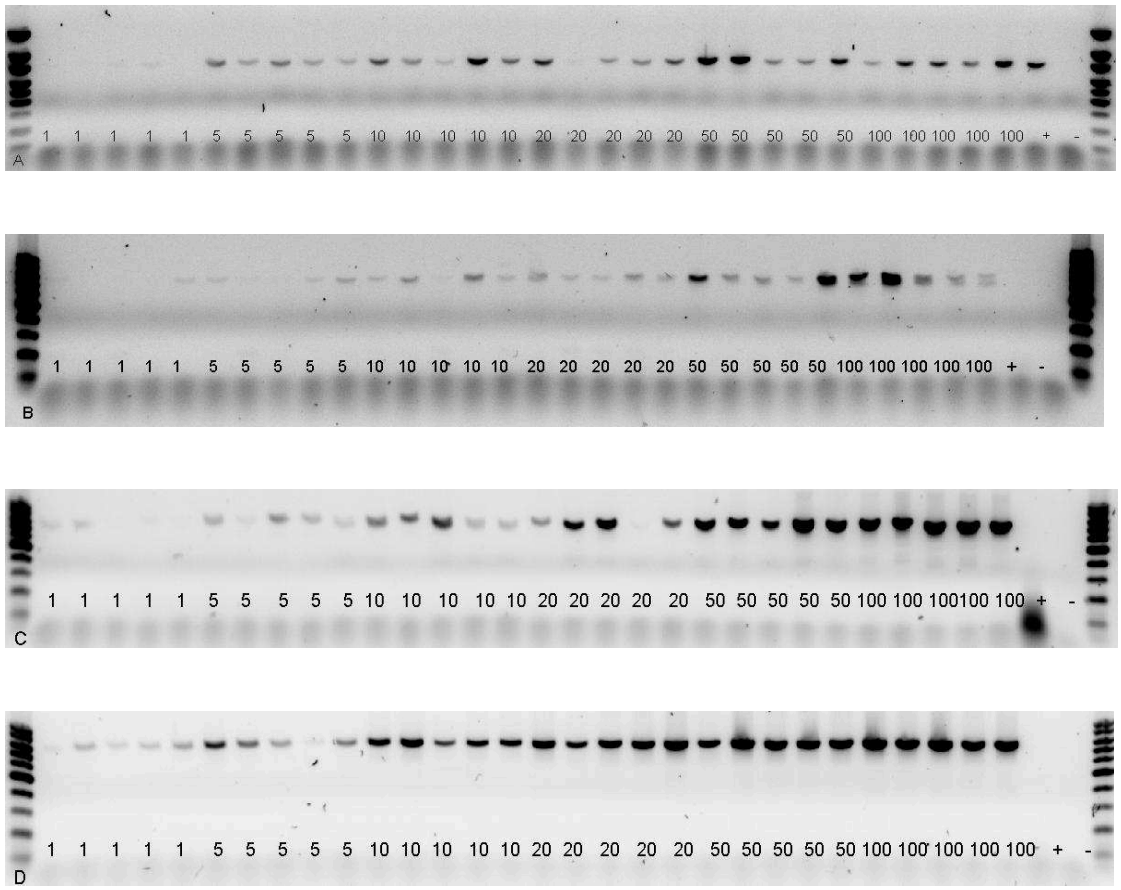

Figure 3.8. Agarose gels of PCR amplicons from STYCLAV18S assay from mussel processing plant effluent water samples **A.** Direct grab mussel processing plant effluent water samples spiked with 1, 5, 10, 20, 50 and 100 S. clava larvae. **B.** Sieve-concentrated musself processing plant effluent water samples spiked with 1, 5, 10, 20, 50 and 100 S. clava larvae. **C.** Direct grab mussel processing plant effluent water samples spiked with 1, 5, 10, 20, 50 and 100 S. clava eggs. **D.** Sieve-concentrated mussel processing plant effluent water samples spiked with 1, 5, 10, 20, 50 and 100 S. clava eggs.

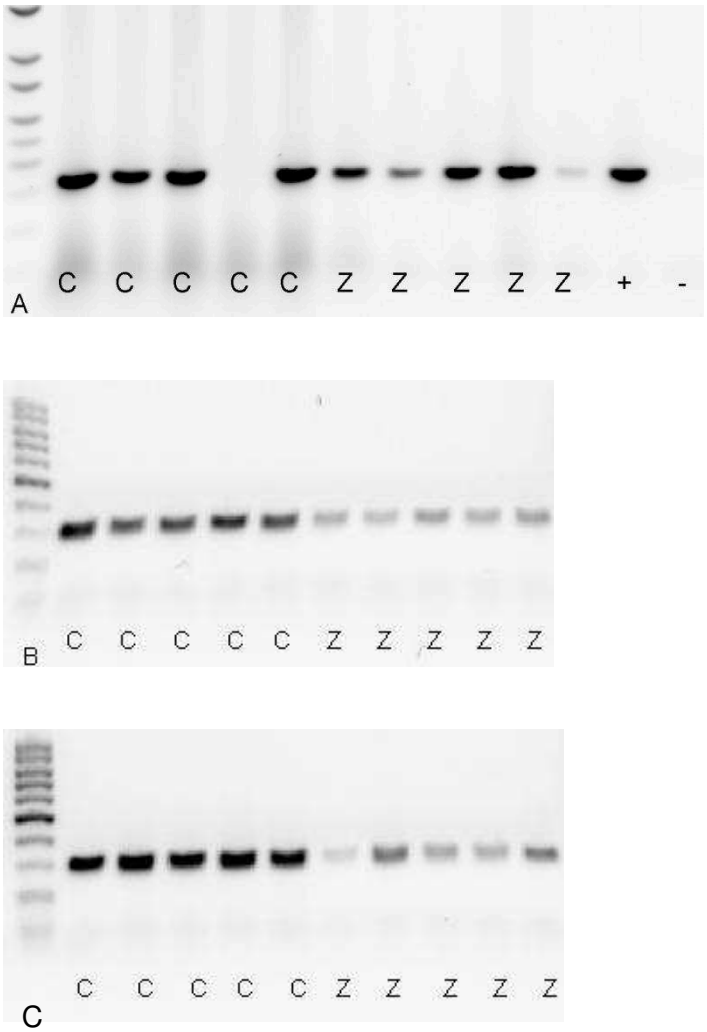

Figure 3.9 Agarose gels of PCR amplicons from BOTSCHLOCOI assay spiked with colony (C) or zooid (Z). **A.** Sieve-concentrated bay water. **B.** Sieve-concentrated mussel processing plant effluent water. **C.** Direct grab mussel processing plant effluent water.

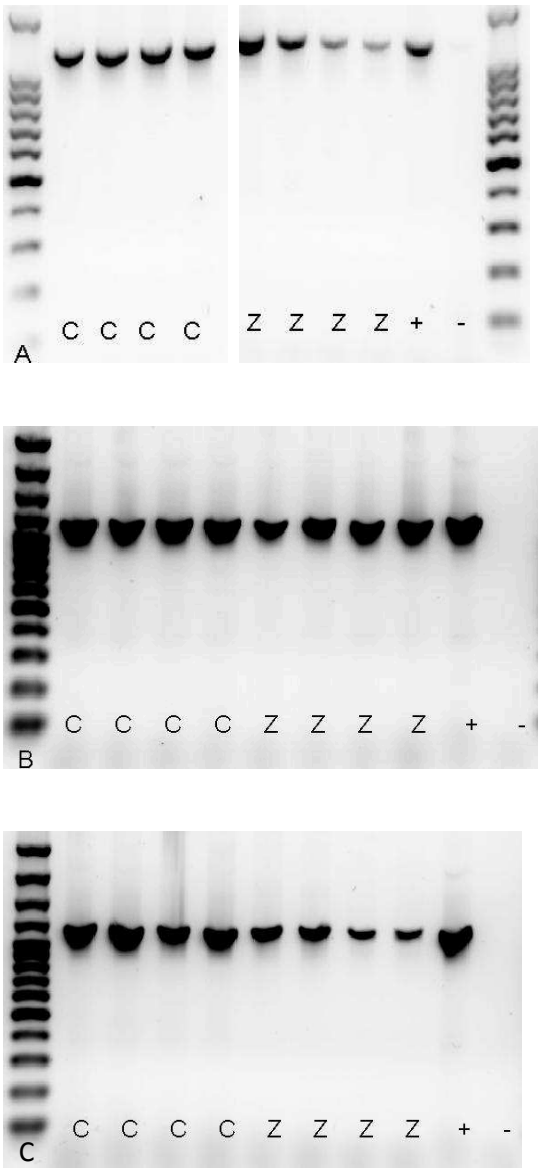

Figure 3.10 Agarose gels of PCR amplicons from BOTVIOLET18S assays from water samples spiked with colony (C) and zooid (Z) of Botrylloides violaceus **A.** Sieve-concentrated bay water samples. **B.** Sieve-concentrated mussel processing plant effluent water samples. **C.** Direct grab mussel processing plant effluent water samples.

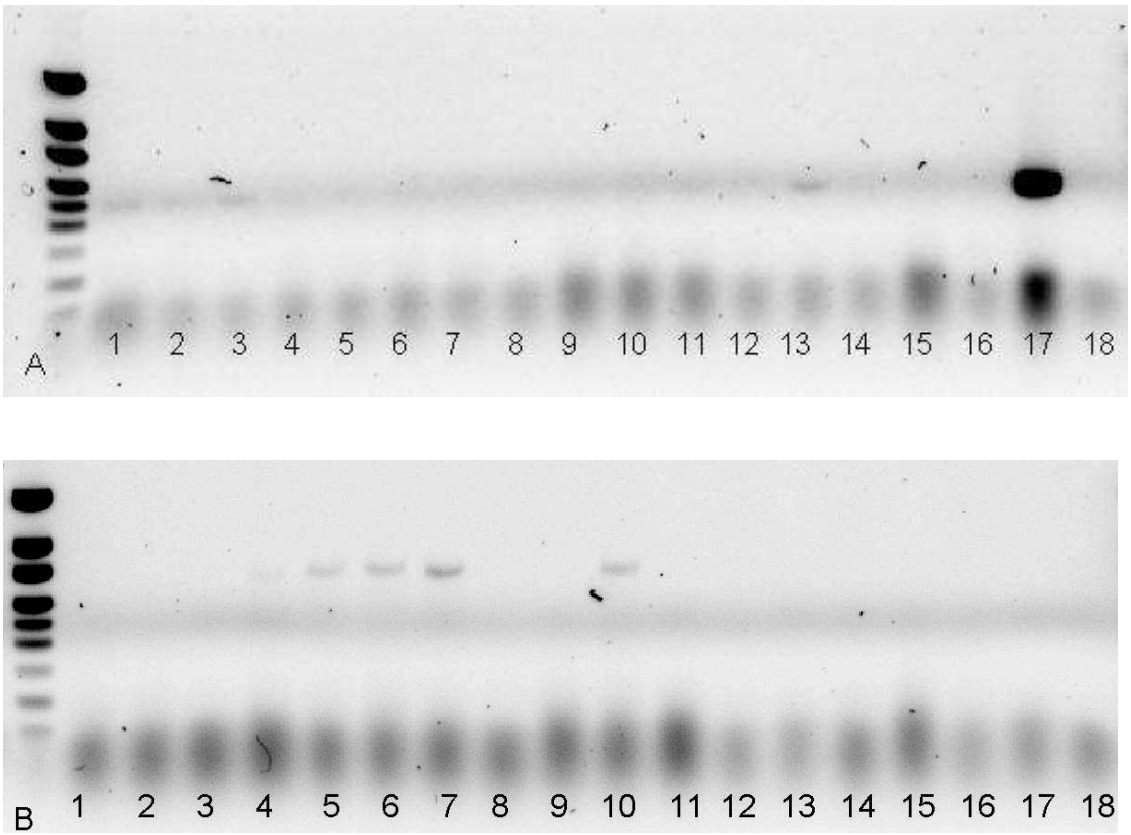

Figure 3.11 A. Agarose gel of PCR amplicons from the CIONAINTESTCOI assay with water samples from PEI. **B.** Agarose gel of PCR amplicons from the STYCLAV18S assay with water samples from PEI. Ln1=Montague River. Ln2= St. Mary's Bay. Ln3= Boughton River. Ln4= Darnley Basin. Ln5=Marchwater. Ln6=Marchwater. Ln7=Marchwater. Ln8=Murray River. Ln9=Cardigan River. Ln10=Darnley Basin. Ln11=Cardigan River. Ln12= Murray River. Ln13=Boughton River. Ln14=Marchwater. Ln15=Cardigan River. Ln16=Murray River. Ln17=Positive Control. Ln18= Negative Control.

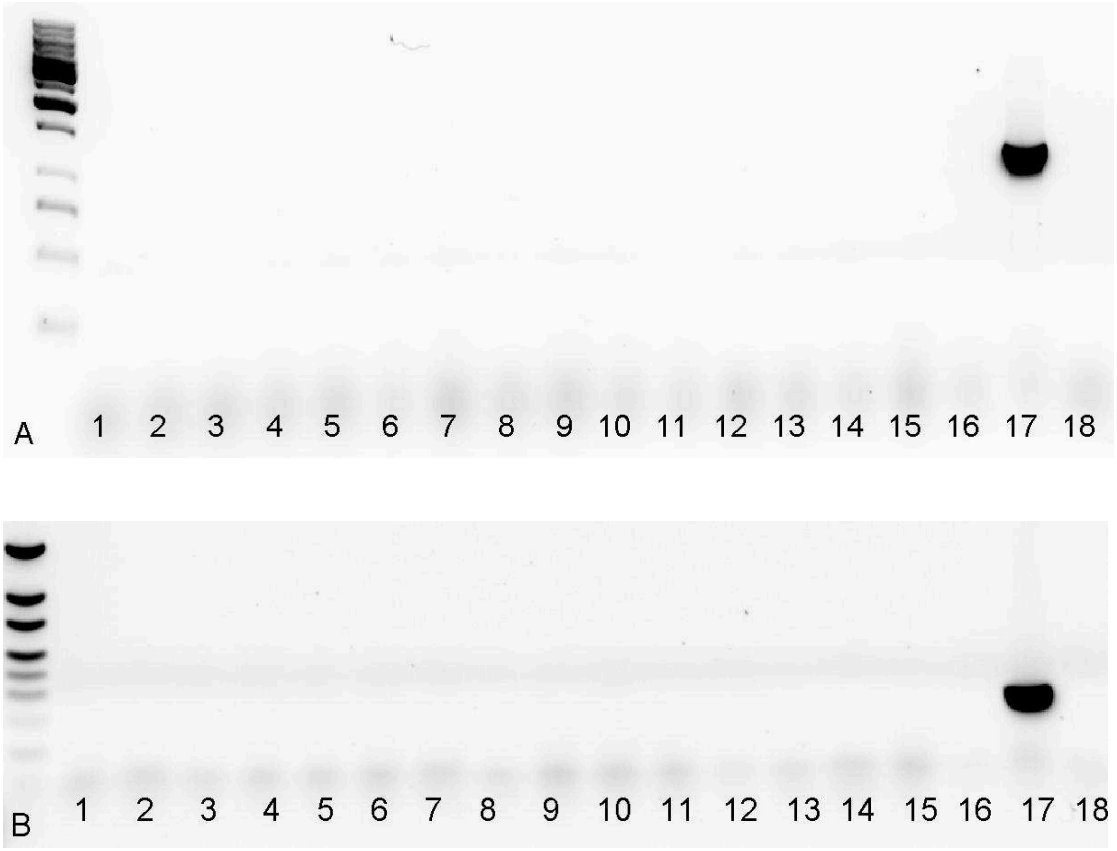

Figure 3.12 A. Agarose gel of PCR amplicons from the BOTVIOLET18S primer sets with water samples from PEI. **B.** Agarose gel of PCR amplicons from the BOTSCHLOCOI primer sets with samples from PEI. Ln1=Montague River. Ln2= St. Mary's Bay. Ln3= Boughton River. Ln4= Darnley Basin. Ln5=Marchwater. Ln6=Marchwater. Ln7=Marchwater. Ln8=Murray River. Ln9=Cardigan River. Ln10=Darnley Basin. Ln11=Cardigan River. Ln12= Murray River. Ln13=Boughton River. Ln14=Marchwater. Ln15=Cardigan River. Ln16=Murray River. Ln17=Positive Control. Ln18= Negative Control.

3.4 Discussion

3.4.1 Optimization of Assay Protocols

DNA Extraction Kits

The Qiagen DNeasy DNA extraction kit was successful in extracting amplifiable DNA from both direct grab and sieve-concentrated bay water samples. However, this kit was not able to extract amplifiable DNA from the mussel processing effluent water, which contains more sediment and PCR inhibitors than the bay water. Qiagen QIAamp DNA extraction kits were also unable to remove inhibitors in other studies in which more specialized soil extraction kits were successful in removing inhibitors from DNA samples (Alexandrino et al. 2004; Behets et al. 2007). While the Qiagen kits do remove some inhibitors during the extraction method, they are not specialized extraction methods developed for use with environmental samples which are high in inhibitory compounds. The Norgen Soil DNA Isolation kit has targeted steps during the extraction procedure which use both chemical and physical methods to separate inhibitors such as humic acids from the nucleic acid sample. These steps were sufficient to remove the PCR inhibitors in the mussel effluent processing water in our study. However, the Norgen Soil DNA Isolation kit did not extract amplifiable DNA from bay water samples. Spectrophotometer analysis showed that DNA was extracted from these samples; however this DNA appeared sheared when run through an agarose gel (Data not shown). The Norgen extraction method includes a step in which samples are vortexed with bead tubes and it appears that in the absence of heavy sediment loads, DNA is sheared during this process. As a result, we recommend using Qiagen DNeasy kits to extract DNA from water samples low in sediment (such as bay water) and Norgen Soil DNA Isolation kits to extract DNA in water samples that are high in sediment (such as mussel processing plant effluent). These results are similar to Behets et al. (2007),

who also determined that Qiagen DNA extraction kits were sufficient in one water sample type but that a second type of water had enough inhibitors to require a specialized soil DNA isolation kit to overcome the inhibition in a diagnostic assay for Legionella pneumophila.

Sample Dilution in PCR

From the dilution study, it is clear that in mussel processing plant effluent water samples, diluting the DNA samples 10-fold prior to adding them to PCR is optimal for assay sensitivity. This indicates that while the Norgen Soil DNA Isolation kit is successful in removing most of the PCR inhibitors from the DNA samples, enough inhibitors remain to have an inhibitory effect on the assay. By diluting the samples with distilled water, the concentration of inhibitors is decreased below the inhibition threshold, allowing amplification to occur despite the fact that the DNA concentration is also diluted in the process. Using dilutions to overcome this inhibition threshold has also been exhibited in other studies (Verkooyen et al. 1996; Kemp et al. 2006). Sample dilution had no significant impact on the Qiagen extracted bay water samples, since these samples did not contain high levels of inhibitory compounds in the initial water samples.

Water sampling Method

Assay sensitivity did not appear to be impacted by water sample collection method, since assay performance was similar in both direct grab and sieve concentrated spiked samples. Since there would be a greater chance of detecting tunicate eggs or larvae in the sieve-concentrated method (due to a higher volume of water being sampled) we would recommend using the sieve-concentrated method of sampling with these assays. This is

particularly important in bay water sampling since the concentration of tunicate eggs and larvae is lower in bay water then in mussel processing effluent water (Bourque et al. 2006; PEI Department of Fisheries, Aquaculture and Rural Development 2009).

3.4.2 Sensitivity analysis with spiked field water samples

In both bay water and mussel processing effluent water, the four species specific assays were very sensitive in detecting eggs, larvae, zooids and colonies of invasive tunicates in this study. The CIONAINTESTCOI assay detected 97% of all quantities of egg and larvae tested in bay water samples this study to a sensitivity of 1 egg or larva. This assay also detected 100% of C. intestinalis eggs and larvae tested in mussel processing effluent to a sensitivity of 5 larvae or eggs. The STYCLAV18S assay detected 98% of all quantities of S. clava eggs and larvae in bay water samples in this study to a sensitivity of 1 egg and 1 larva. This assay also detected 100% of S. clava eggs and larvae in mussel processing plant effluent sample to a sensitivity of 5 larvae. The BOTSCHLOCOI assay detected B. schlosseri in 90% of bay water samples and 100% of mussel processing plant effluent to a sensitivity of 1 zooid. The BOTVIOLET18S assay detected B. violaceus in 100% of bay water and mussel processing plant effluent water to a sensitivity of 1 zooid. The high sensitivity in these four assays is comparable to other assays developed to detect larvae of invasive species such as 1-5 larvae in Limnoperna fortunei, (Boeger et al. 2007), 1 cyst in Gymnodinium catenatum (Patil et al. 2005) and 1 zoea in Carcinus sp. (Darling & Tepolt 2008).

3.4.3. Comparison with Industry Standard

Both microscopy and molecular analysis are in congruence with the negative results for both colonial species of tunicate (B. schlosseri and B. violaceus) in each river and bay tested for invasive tunicates in this study. These negative values are not surprising as the colonial tunicates are not frequently found in large numbers in these regions. In fact, during the weekly May-November testing of these rivers and bays by the PEI Department of Fisheries, Aquaculture and Rural Development, only one bay (St. Mary's) tested positive for colonial tunicate larvae on one collection date. The STYCLAV assay detected S. clava in 2/3 of the positive microscope samples. The CIONAINTESTCOI assay detected C. intestinalis in 4/5 of the positive microscope samples. Larval content varied from 1-58 in the water samples from different locations. The molecular assays detected target material to a limit of 3 larvae (St. Mary's Bay); however, the molecular assays had negative results for two samples that each contained 1 larva as determined by the microscope analysis. This slight decrease in sensitivity between the two methods is likely due to the fact that the microscope analysis occurred first while samples were fresh. Following analysis with the microscope, samples were stored for up to two days at -4^{o}C in tap water prior to molecular analysis. Exposure to tap water and freezing at -4^{o}C may have led to nucleic acid degradation. In addition the few larvae detected during the microscope analysis may not have all been recovered from the counting wheel when samples were washed back into sample tubes for molecular analysis. These experimental limitations and time delays were due to the necessity of utilizing the water samples collected by the Department of Fisheries, Aquaculture and Rural Development and by the need to compare the same water samples with the two methods of analyses. In future, when the molecular

assays are used in field water monitoring programs to detect invasive tunicates, all samples should be processed fresh or snap frozen to ensure optimal sensitivity.

The high throughput capacity and efficiency of the PCR based assays developed in this study are important characteristics to note when comparing the industry standard microscope analysis of water samples with the molecular assays in detecting invasive tunicate eggs and larvae. The Department of Fisheries, Aquaculture and Rural Development estimates that it requires 30 minutes to assess tunicate content in each bay water sample using traditional microscope analysis (Aaron Ramsay, personal communication). Due to the high sediment content in mussel processing plant effluent, the Department of Fisheries and Oceans estimates that it requires 2-4 hours to assess tunicate content in each effluent water sample using traditional microscope analysis (Daniel Bourque, personal communication). In contrast, 96 bay water samples can be processed per day using the molecular assays designed in this study, and 96 effluent water samples can be processed per 1 ½ days using the molecular assays to assess tunicate content. This high throughput capacity of the PCR assays, in addition to their sensitivity and specificity, make the assays ideal tools for large scale surveillance programs that monitor for invasive tunicate eggs and larvae in water surrounding PEI.

3.4.4 Conclusion

Invasive species management requires surveillance and monitoring programs that have the capacity to detect invasive species in new regions before population levels become too large to control (Mehta et al. 2007). Increasingly, PCR is being used to detect aquatic invasive species eggs and larvae in surface and ballast water samples (Patil et al. 2005;

Boeger et al. 2007; Darling & Tepolt 2008) since large quantities of water can be screened for trace amounts of eggs and larvae in a high throughput manner. PCR assays are also powerful diagnostic techniques because the technology and equipment are now available in any molecular biology laboratory making developed assays accessible to many regions. As the results of this study show, PCR assay efficacy can be altered in different water sample types, and it is critical that molecular assays be validated in each specific situation in which they are used. Characteristics of the water samples, such as the presence of PCR inhibitory compounds and the presence of non-target organisms can impact assay sensitivity. As this study shows, inhibition can be overcome by troubleshooting extraction methods and PCR protocols. Since molecular assay performance is also dependent on the specificity of the primer sequence, haplotypes in different geographic regions may also impact assay efficacy. For this reason, assays should also be validated when used in new geographic regions.

This study has demonstrated the powerful sensitivity of the four species specific invasive tunicate assays to detect 1-5 eggs and larvae in both bay water and mussel processing plant effluent water in Prince Edward Island. Both the sensitivity and repeatability of the four assays make them excellent candidates for invasive species monitoring programs. It is hoped that these assays can be integrated into monitoring programs for invasive tunicates in Prince Edward Island since early detection is critical in invasive species control and management.

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Chapter 4: Temporal gene expression as a marker for early life stages of Ciona intestinalis in water samples as well as a viability marker for larvae

4.1 Introduction

Ciona intestinalis (Vase Tunicate), is one of four invasive tunicates which is currently causing significant fouling problems to the mussel aquaculture industry in Prince Edward Island. Of the four invasive tunicates, Ciona intestinalis is the species that poses the greatest threat to the mussel aquaculture industry due to the weight of fouling that this species exhibits on aquaculture lines and gear (Ramsay et al. 2008; Ramsay et al. 2009). C. intestinalis has become the dominant tunicate species in many of these rivers due to its ability to reproduce at lower temperatures, faster rate of growth, tolerance to high density crowding and its mucoidal tunic which inhibits settlement from other tunicate species (Ramsay et al. 2008; Ramsay et al. 2009). Although Carver et al. (2003) demonstrated that Ciona intestinalis produced gametes in April at 4^{o}C, Ciona intestinalis recruitment was not observed until mid-June at 8^{o}C (Ramsay et al. 2008). This reveals that although gametes may be detected in water samples, this does not necessarily mean that viable larvae will also be present. It is critical for mussel aquaculture growers to know when viable larvae are present in their aquaculture lease areas, so that they can plan treatment protocols accordingly.

Although the molecular assay CIONAINTESTCOI developed in chapter 2 and field tested in chapter 3 can detect the presence of Ciona intestinalis tissue in water samples, this assay cannot distinguish between different life stages of this invasive species. This is due to the fact that the species assays detect DNA, which is present at all life stages of the individual tunicate (Burreson 2000). While each cell of an organism contains copies of identical DNA

molecules through all stages of development, mRNA content and quantity varies throughout different stages of development (Azumi et al. 2007). Therefore to distinguish between different life stages in Ciona intestinalis, mRNA based detection assays could be developed. Such an assay could be implemented in surveillance and monitoring programs to screen for eggs and larvae of Ciona intestinalis in bay water, in mussel processing plant effluent water and in ballast water. While other studies use qRT-PCR assays to detect infectious agents such as arenaviruses (Grajkowska et al. 2009) foot and mouth disease viruses (Huang et al. 2009) rabies virus (Nadin-Davis et al, 2009) Mourilyan virus (Cowley et al. 2009) and cancerous markers such oral leukoplakia and oral squamous cell carcinomas (Kondoh et al. 2007), circulating tumour cells in metastatic breast cancer (Van der Auwera et al. 2009) and CD39 expression in pancreatic cancer cells (Loos et al. 2010), no study has used qRT-PCR as a diagnostic assay for different life stages of an organism. Since the genome of Ciona intestinalis was sequenced (Dehal et al. 2002), extensive analysis has occurred measuring gene expression in Ciona intestinalis across many life stages during development (Hotta et al. 2000; Nishikata et al. 2001; Satou et al 2002; Chiba et al. 2003; Hino et al. 2003; Ishibashi et al. 2003; Imai et al 2004; Yagi et al. 2004; Kawashima et al. 2005; Deyts et al. 2006; Miwata et al. 2006; Yamada 2006; Azumi et al. 2007; Comes et al. 2007; Hamaguchi et al 2007; Hotta et al. 2007; Imai & Meinertzhagen 2007; Matsumoto et al. 2007; Sekiguchi et al. 2007). From these studies, it is clear that temporal gene expression changes throughout the developmental life stages of C. intestinalis (Satou et al. 2002; Kawashima et al. 2005; Azumi et al. 2007). Based on EST data, Kawashima et al. (2005) showed that 25% of developmental genes in Ciona intestinalis are expressed at multiple points during development while the remaining 75% are either single use genes or genes that are constantly expressed across all developmental

life stages. The main objective of this study is to screen the expression of selected genes in egg, larvae and adult stages of Ciona intestinalis with qRT-PCR in order to develop life stage specific molecular markers for the egg and larval life stages of Ciona intestinalis.

The life stage specific genes targeted in this study were selected based on fluorescence in situ hybridization (FISH), whole-mount in situ hybridization (WMISH), EST, and microarray studies involving multiple life stages of Ciona intestinalis (Yoshida et al. 1997; Chiba et al. 2003; Ikuta et al. 2004; Imai et al. 2004; Yamada et al. 2005; Miwata et al. 2006). From these studies, six genes were chosen as potential life stage markers for free swimming larvae: Homeobox-2 (HOX2) (Ikuta et al. 2004), Homeobox even-skipped homolog B (EvxB) (Ikuta et al. 2004), Actin-1 (MA1), Tropomyosin-2 (TPM2) (Chiba et al. 2003), Tropomyosin-1 and CA3 (Satoh 2000). In addition, four genes were screened as potential life stage markers for unfertilized eggs: PEM Homeobox gene (PEM) (Yoshida et al. 1997), PEM-13 Homeobox gene (PEM-13) (Yamada et al. 2005), Zinc Finger 364 (ZF364) and Zinc Finger 054 (ZF054) (Miwata et al. 2006).

An integral component of qRT-PCR assays are normalisation genes that act as internal controls across all experimental variables (Bustin 2002). Normalisation genes are used to correct for variation in gene expression which may exist within the experimental procedure such as between individual samples, different reagents, extraction procedures, presence of inhibitors and inefficiencies in assay runs. If assays have appropriate internal controls, treatment effects can be detected despite any variation due to experimental effects. A defining characteristic of appropriate normalisation genes is that they must exhibit stable

gene expression across all variables within each experiment. Since numerous studies have now shown that no one gene is suitable as a universal normalisation gene across all possible experimental conditions, appropriate normalisation genes must be validated in each experimental situation (Bustin et al. 2005). In addition, qRT-PCR assays must no longer involve only one normalisation gene, but instead include several normalisation genes (Vandesompele et al. 2002; Bustin et al. 2005; Nolan et al. 2006; Peters et al. 2007). Several normalisation genes have been used in other qRT-PCR experiments involving Ciona intestinalis including RPS18, RPL11, (Olinski et al 2006) RPS27A (Olinski et al. 2006; Comes et al. 2007), GAPDH (Olinski et al. 2006; Coric et al. 2008) calmodulin (CiCAM) (Piscopo et al. 2000; Matias et al. 2005) and α-tubulin (DeLigio & Ellington 2006). The second objective of this experiment is to screen the gene expression levels of these six normalization genes to determine which exhibit stable gene expression across the life stages involved in this study. In this study, normalisation genes will be used as positive controls rather than normalisation factors, since gene expression levels are not being compared across the three life stages. Instead, genes are being screened for exclusive expression at each life stage, so although no expression may be seen for the target gene at all other life stages, the expression of normalisation genes will indicate that this is due to the fact that the target transcript is not present in the sample and not that the sample has degraded.

As indicated above, the target molecule for the life stage specific assays developed in this study are mRNA transcripts. mRNA transcripts have varying levels of stability depending on the gene, with some mRNA transcripts degrading within minutes of transcription and

others remaining stable for up to 24 hours (Tourrière et al. 2002). The stability of an mRNA transcript is often related to the duration that its translated protein is required in the cell. Proteins that are quickly produced in response to developmental or external cues are often from mRNA transcripts with short half-lives whereas proteins that are present in stable concentrations over a long time period are generally from mRNA transcripts with long half-lives (Guhaniyogi & Brewer 2001). For gene regulation purposes, mRNA transcripts are often degraded by endonuclease and exonuclease activity (Tourrière et al. 2002). In addition post-death, mRNA is rapidly degraded by RNases (Fontanesi et al. 2008). This characteristic may allow for some mRNA transcripts with short post-mortem half-lives to be used as viability assays in Ciona intestinalis larvae. The degradation of mRNA transcripts post-mortem has been the focus of many recent studies. These studies have shown that mRNA degradation post-mortem is gene dependent, with some mRNA transcripts degrading within minutes of death, and others stable after 48 hours (Zhao et al. 2006; Fontanesi et al. 2008) and even 96 hours (Yasojima et al. 2001). It would be important for surveillance and monitoring programs, especially in programs screening ballast water or mussel processing plant effluent water, to be able to determine whether the larvae being detected in water samples are viable or nonviable organisms. This could be determined by targeting mRNA transcripts that are quickly degraded post-mortem. The third objective of this study is to evaluate the transcript stability of life stage specific markers generated in this study to determine whether any of the target genes could be markers for larval viability.

4.2 Materials and Methods

4.2.1. Tissue collection

Ciona intestinalis samples were collected from Murray River PEI, from July-August 2007. Eggs were collected by piercing the oviduct with a syringe needle and eggs were removed using a pipette. Eggs were immediately placed in 1.5ml tubes containing 200µl RNAlater and were incubated overnight at 4^{o}C. Each tube contained pools of eggs from 3 individuals. Tubes were then centrifuged at 2000 x g for 2 minutes and RNAlater was removed from the tubes with a pipette. The tubes containing eggs were then snap frozen in liquid nitrogen and stored at -80^{o}C.

Free swimming larvae were generated in the lab by removing egg and sperm samples from multiple Ciona intestinalis individuals. These gametes were then placed in separate graduated cylinders containing 200ml of filtered sea water before being mixed together by swirling. Gametes were then poured into petri dishes with filtered sea water and left for 18 hours. Free swimming larvae were individually collected by pipette and placed in microcentrifuge tubes in pellets ranging from 111-364 free swimming larvae. Tubes containing free swimming larvae were immediately snap frozen in liquid nitrogen and stored at -80^{o}C. Free-swimming larvae were not placed in RNAlater due to the results of preliminary experiments which showed that the larvae float on top of the RNAlater solution and do not pellet making later removal of the RNAlater solution from the free-swimming larvae impossible.

Three adult specimens were used to collect adult tissue. The tunic was first removed from each specimen via dissection with a scalpel blade. Individual specimens were then finely

chopped with a scalpel blade and tissues were mixed together to ensure that each sample contained multiple adult tissues. Tissue mixtures from each individual were divided into four 30 mg quantities for later RNA extraction and were placed in 1.5ml microcentrifuge tubes. Adult samples were then snap frozen in liquid nitrogen and stored at -80^{o}C until RNA was extracted.

4.2.2. RNA Extractions

All RNA extractions were performed on egg, free swimming larvae and adult tissues using Qiagen RNeasy Minikits (Qiagen Inc, Canada). Extractions were all performed following manufacturer's instructions. For each of the three adult specimens, RNA from all four tubes per individual was pooled prior to cDNA synthesis so that RNA from the entire individual would be available for each of the three adult cDNA synthesis.

4.2.3. cDNA Synthesis

cDNA synthesis was performed using 100ng RNA from each sample. Qiagen Quantitect RT kits were used for all cDNA synthesis reactions and all reactions were conducted according to manufacturer's protocol.

4.2.4 qPCR Primer Design

All life stage specific primers were manually designed from EST sequences obtained from GenBank and from Ghost Database (http://ghost.zool.kyoto-u.ac.jp/indexr1.html) and JGI Ciona intestinalis V1.0 (http://genome.jgi-psf.org/ciona4/ciona4.home.html)and from ANISEED (http://crfb.univ-mrs.fr/aniseed/) (See Table 4.1). To minimize false-positives, all primers were assessed to ensure specificity by NCBI-BLAST (National Centre for

Biotechnology Information- Basic Local Alignment Search Tool) (Altschul et al. 1997). Primer suitability was further evaluated using IDT Oligoanalyzer 3.0. Normalisation gene primers were all obtained from other studies: RPS18, RPL11, (Olinski et al 2006) RPS27A (Olinski et al. 2006; Comes et al. 2007), GAPDH (Olinski et al. 2006; Coric et al. 2008) calmodulin (CiCAM) (Piscopo et al. 2000; Matias et al. 2005) and α-tubulin (DeLigio & Ellington 2006) (See Table 4.2).

4.2.5. qPCR

Quantitative analysis of RNA levels was performed using iTaq SYBR Green Supermix with ROX (Bio-Rad, Canada) on a Chromo4™ Real-time detector (Bio-Rad, Canada). Each sample was run in duplicate or triplicate and average Ct values were calculated for each sample. Every sample was run in three serial dilutions to ensure that reaction efficiencies were appropriate. Two normalisation genes (RPL11 and RPS27A) were monitored in every run to ensure that target sample levels were not significantly altered during extraction, cDNA synthesis and amplification procedures. No template controls were included in each run to ensure levels were not impacted by contamination. No-RT controls were run with most samples to ensure that samples were not contaminated with genomic DNA. Ct values for each target gene were only compared to samples run in the same run to reduce between run variations in fluorescence levels. Melting curves were calculated and evaluated in every run to ensure that primer dimers were not contributing to fluorescence levels. Normalisation genes were screened using samples from all adult and free swimming life stages and results were analyzed using BestKeeper-1 (Pfaffl et al. 2004).

Table 4.1 qPCR primers designed in this study for life-stage specific assays for Ciona intestinalis eggs and larvae

Primer Name	Sequence 5'-3'	GenBank Accession Number Or JGI C. intestinalis V1.0 region
MAF	CTGCTGGAATCCACGAG	AK115759
MAR	GGTGGACAATAGATGGGC	AK115759
CA3F	GAAAGGAGGGTTTCAGGAG	XM_002129028
CA3R	GATCCTCCAGCAAGAACG	XM_002129028
TPM1F	AGGACCCAGACACTTTGG	NM_001032538
TPM1R	CCCAGTAGCTTTGTCTTCG	NM_001032538
TPM2F	GAATTGGAATCTGCCCAAG	JGI Ciona intestinalis v1.0 Scaffold_22:380810-383651
TPM2R	TATAAACCCCAATCCCACC	JGI Ciona intestinalis v1.0 Scaffold_22:380810-383651
HOX2F	GGCGCATCCAGGAGACTACG	AB210494
HOX2R	CGTCGGCGCTTGTTACGTCAG	AB210494
ZF054F	CGCATCACTCTTACATCCACATCAATGAGA	XM_002130176
ZF054R	TTTACTCCTGTATTGTCAAACTCCCCATT	XM_002130176
ZF364F	GGTGGAAGCAAAGGTCTGCAC	AK115172
ZF364R	GCACCATTATCCCTCAATCCCCTGG	AK115172
EvxBF	TAGAACTCTACATCTCTGTCGCACCT	AK174659
EvxBR	CGAACTCTGCTATGTAGTACAAACTGGTAC	AK174659
PEMF	GACCTCGACCCCGCAGAG	AK113383
PEMR	TAGCGGTCACACGGCGTG	AK113383
PEM13F	GTGCGGCAATCTGATGCGAGG	BW275407
PEM13R	CTTTGGGTGTGAAGGGCAGTT	BW275407

Figure 4.2 qPCR primers screened as normalisation genes for life-stage specific assays

Primer Name	Sequence 5'-3'	Reference
RPS18F	GAATCGGCCGCAGGTTTAG	Olinski et al. 2006
RPS18R	CCGGCGCGTTTCGTAA	Olinski et al. 2006
α-TubulinF	TGAGCCCTACAACTCCATCC	DeLigio & Ellington 2006
α-TubulinR	CAAAGCACCATCGAATCTCA	DeLigio & Ellington 2006
RPS27AF	GAATCGGCCGCAGGTTTAG	Olinski et al. 2006
RPS27AR	CCTTCCTTATCCTGAATCTTTGCT	Olinski et al. 2006
CAMF	GTTGATGCTGACGGCAACG	Piscopo et al. 2000
CAMR	TCAATCAGCCTATGGAATGA	Piscopo et al. 2000
GAPDHF	GCACTCGTACACTGCTACCCAGAAGAC	Olinski et al. 2006
GAPDHR	GCTGTATCCAAATTCATTGTCGTACCAG	Olinski et al. 2006

4.2.6. Transcript Stability Analysis

Free swimming larvae were generated as previously described (section 4.2.1). Free swimming larvae were then placed in petri dishes filled with fresh water. Previous experiments determined that 2 minute exposures to fresh water were sufficient to kill free swimming larvae of Ciona intestinalis. Following the 2 minute exposure, 100 larvae were sampled and immediately snap frozen in liquid nitrogen. 100 larvae were then sampled and snap frozen at 30 minutes post exposure, 1,2,4,8, and 24 hour periods post exposure. RNA was then extracted and cDNA synthesized as described above. qPCR was performed as described above. Ct levels were compared to evaluate whether there was a change in transcript levels at the different post-mortem intervals.

4.3 Results

4.3.1. Normalisation Gene Screening

Of the six genes screened as normalisation genes in Ciona intestinalis adults and free swimming larvae, only two were selected by BestKeeper as appropriate normalisation genes: RPL11 and RPS27A (Figure 4.1). The gene expression of all other candidate normalisation genes was too variable (Figure 4.2-3) and had CP standard deviations > 1.0 which is the cut-off for genes in BestKeeper.

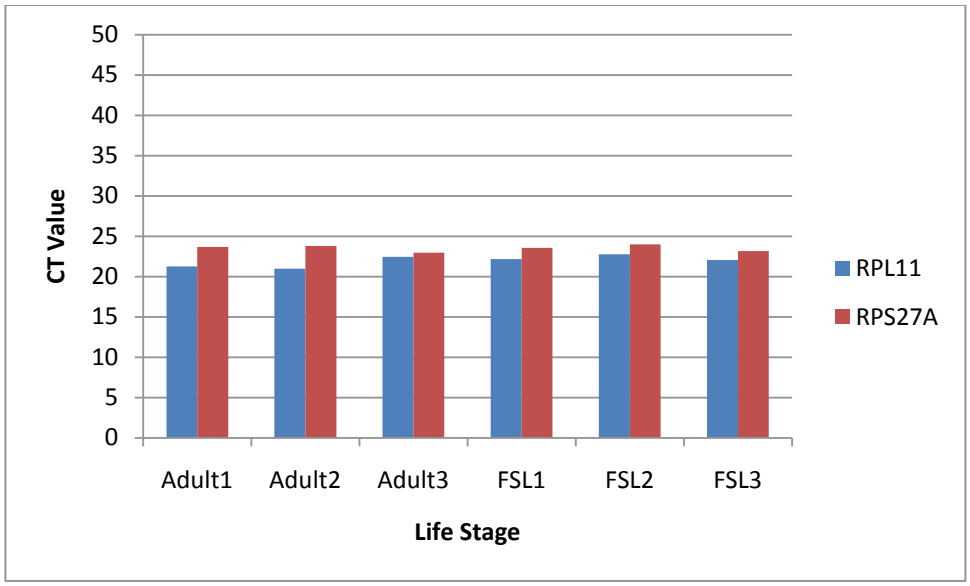

Figure 4.1 Gene expression levels of RPL11 and PRS27A genes in adult and free swimming larvae life stages of Ciona intestinalis.

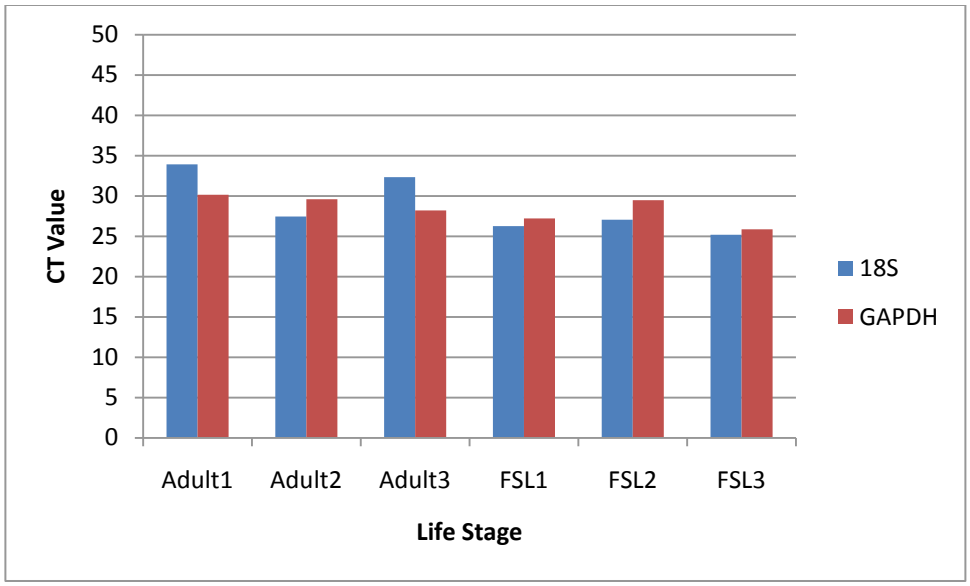

Figure 4.2 Gene expression levels of RPS18 and GAPDH in adult and free swimming larvae life stages of Ciona intestinalis.

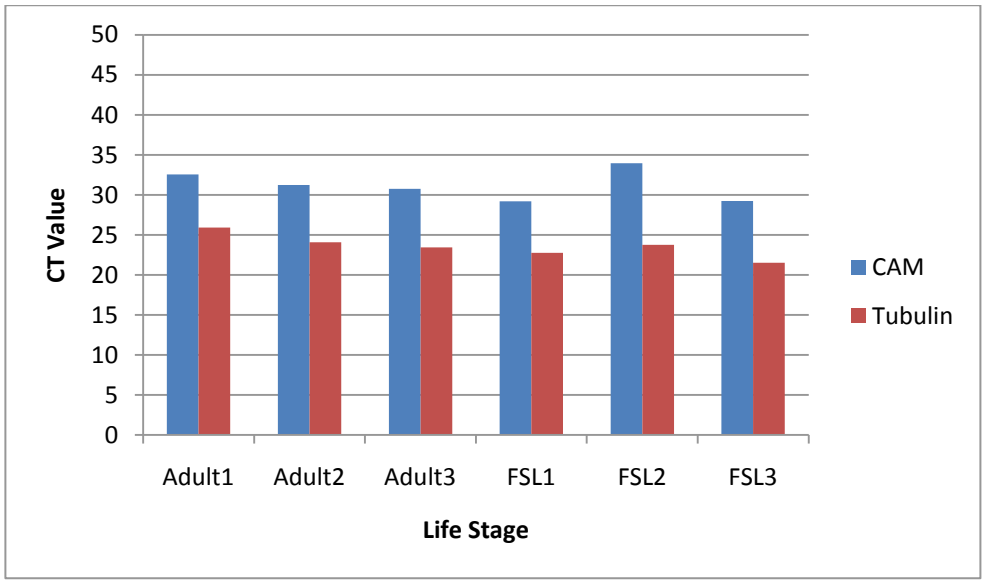

Figure 4.3 Gene expression levels of CAM and α-tubulin genes in adult and free swimming larvae life stages of Ciona intestinalis.

4.3.2. Life Stage Marker

Egg Life Stage

Gene expression of PEM and PEM-13 was detected in all unfertilized egg and free-swimming larvae samples (Figures 4.4-5). ZF364 gene expression was detected all unfertilized egg samples and in 40% of free swimming larvae samples (Figure 4.6). ZF054 gene expression was not detected at any of the life stages in this study although the two normalisation genes RPS27A and RPL11 were detected in all samples (Figure 4.7).

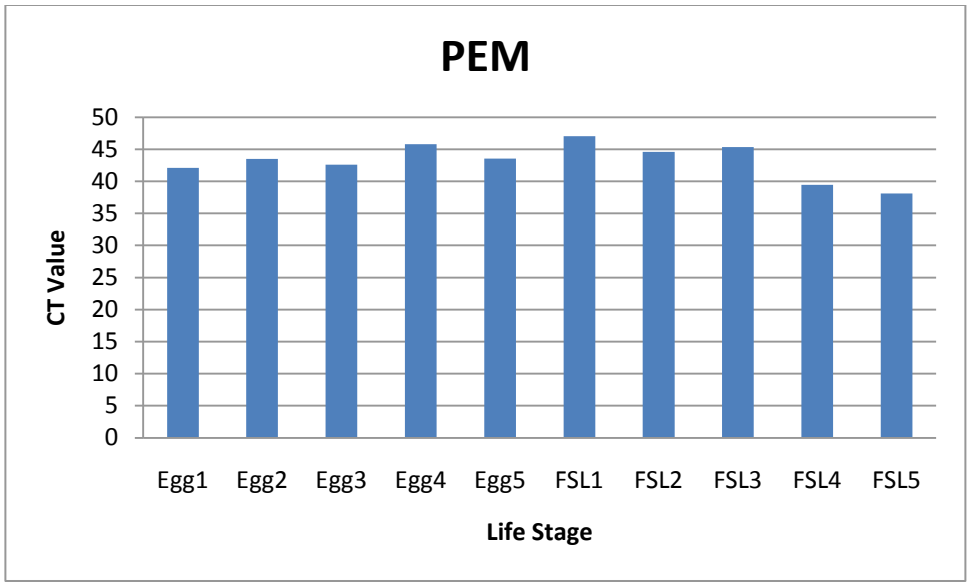

Figure 4.4. Gene expression of PEM at egg and free swimming larvae life stages of Ciona intestinalis.

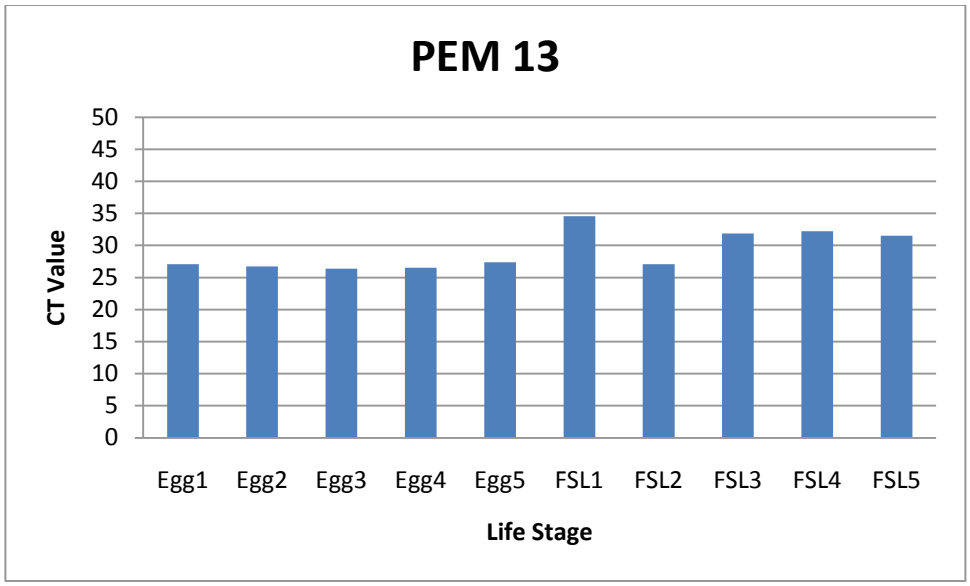

Figure 4.5. Gene expression of PEM13 at egg and larvae life stages of Ciona intestinalis.

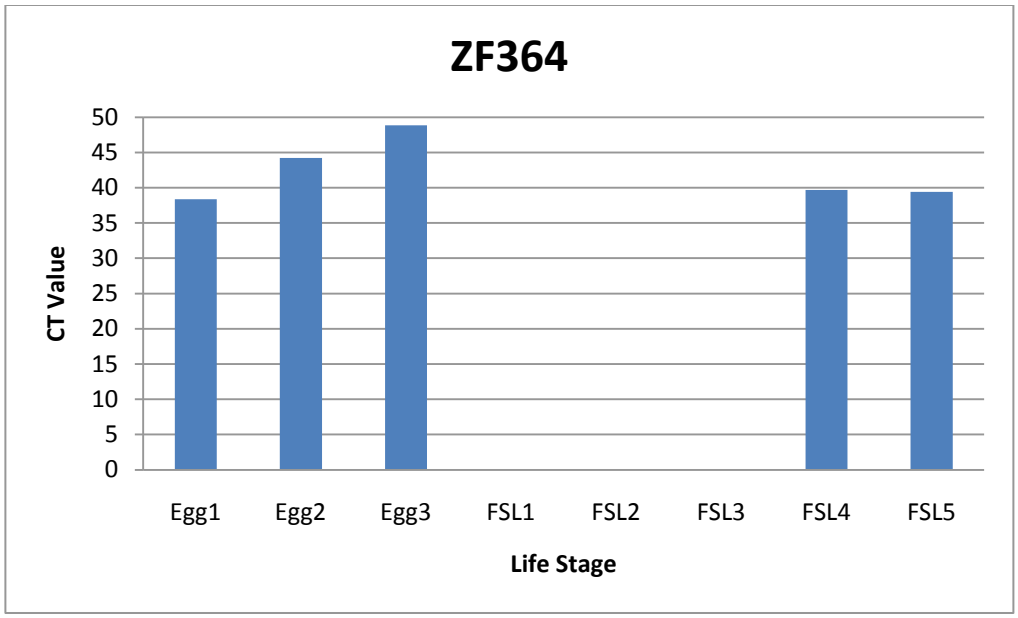

Figure 4.6 Gene expression of ZF364 in egg and free swimming life stages of Ciona intestinalis.

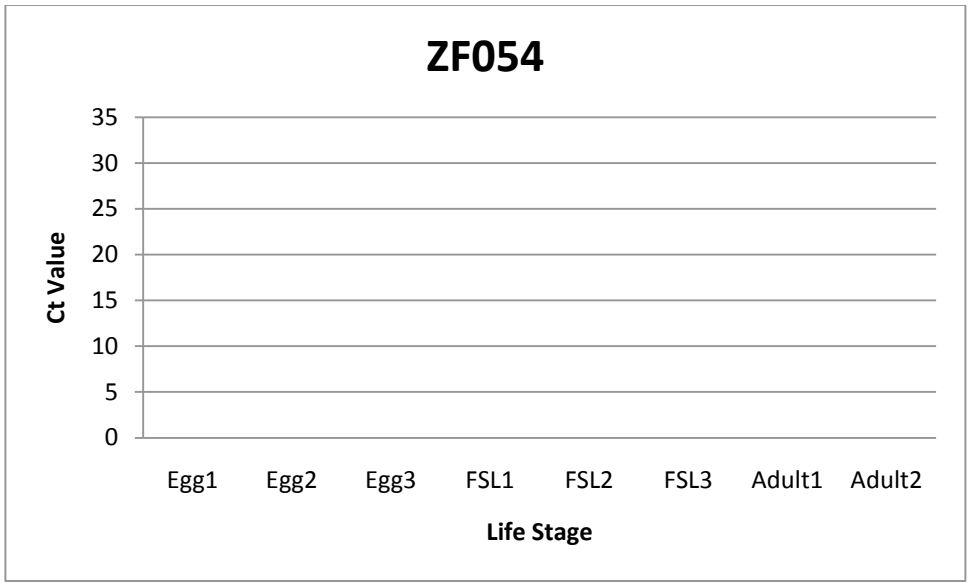

Figure 4.7. Gene expression of ZF054 in egg and free swimming life stages of Ciona intestinalis.

Free swimming larvae life stage

TPM2 gene expression was not detected in adult samples, but was detected in every free swimming larvae sample (Figure 4.8). TPM2 gene expression was also detected in one of the unfertilized egg samples. HOX2 gene expression was detected in all free swimming larvae and adult samples, but was not detected in any of the egg samples (Figure 4.9). MA1 gene expression was detected in every sample across all three life stages (Figure 4.10). TPM1 gene expression was detected in free swimming larvae and adult life stages, but was not detected in any of the egg samples (Figure 4.11). CA3 expression was detected in every sample across all three life stages (Figure 4.12).

4.3.3. Transcript Stability Experiment

Both HOX2 and TPM1 gene expression remained stable 24 hours post mortem (Figure 4.13-14). TPM2 gene expression was last detected 4 hours post-mortem (Figure 4.15). In all three cases, normalisation gene expression (RPS27A & RPL11) remained stable 24 hours post-mortem.

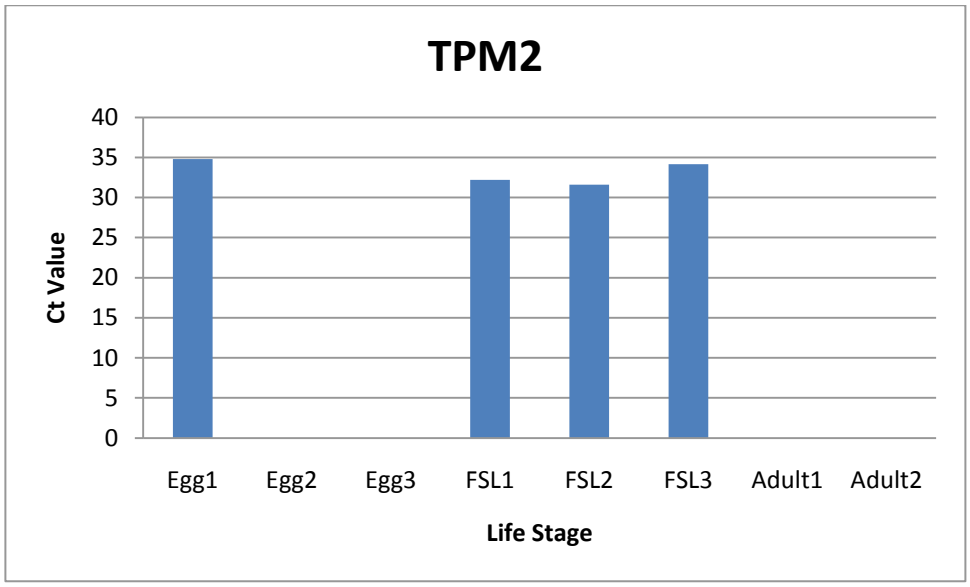

Figure 4.8 Gene expression of TPM2 gene in egg, larvae and adult life stages of Ciona intestinalis.

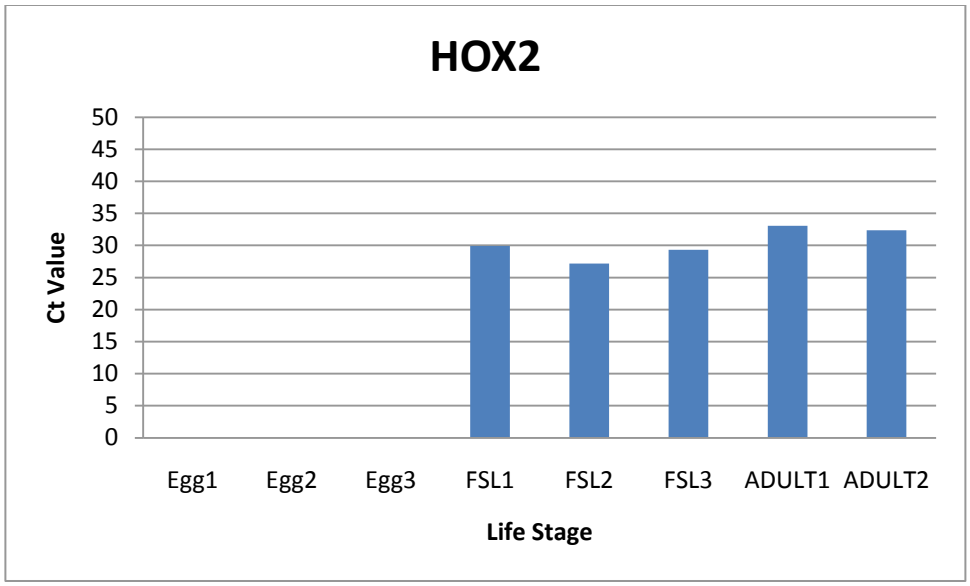

Figure 4.9 Gene expression of HOX2 gene in egg, free swimming larvae and adult life stages of Ciona intestinalis.

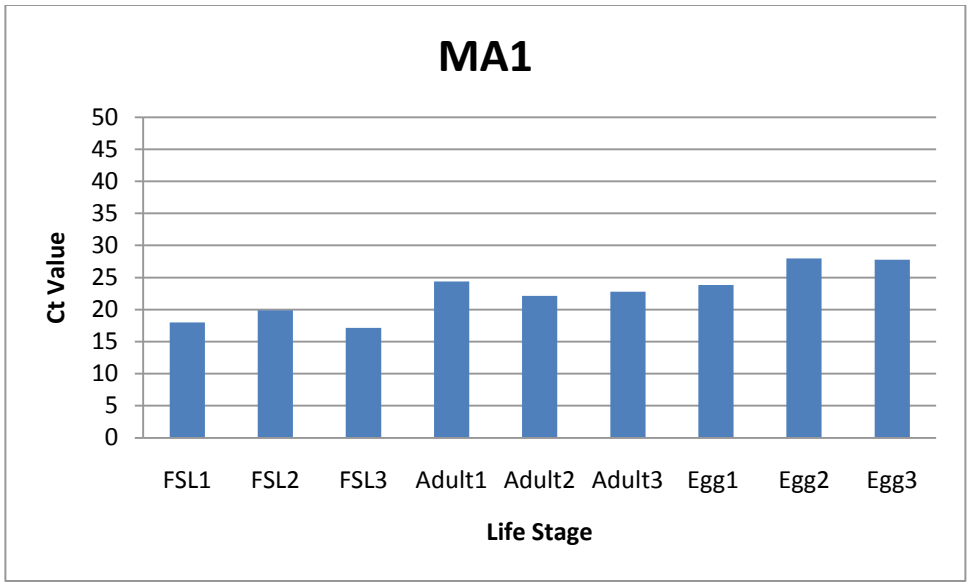

Figure 4.10. Gene expression of MA1 gene in free swimming, adult and egg life stages of Ciona intestinalis.

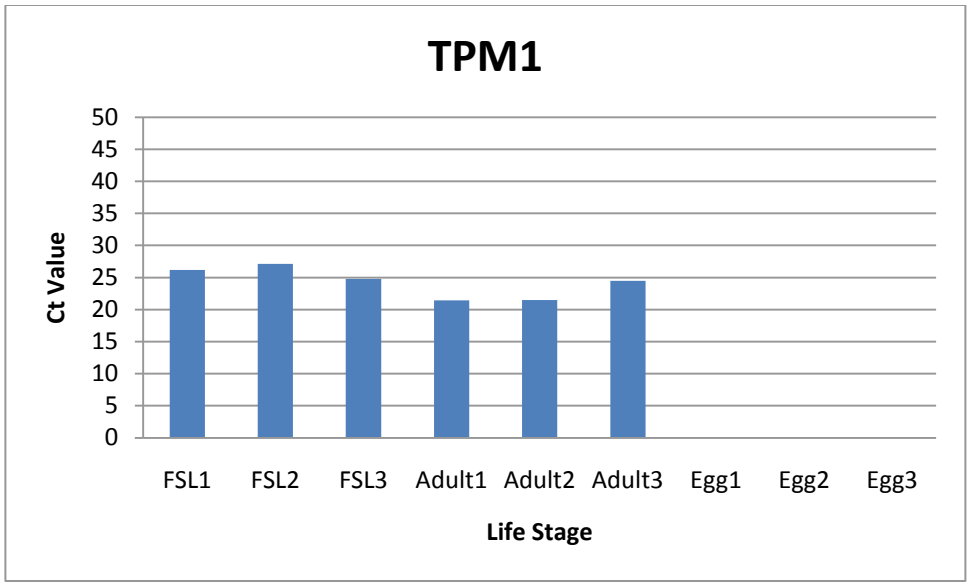

Figure 4.11 Gene expression of TPM1 gene at free swimming larvae, adult and egg life stages of Ciona intestinalis.

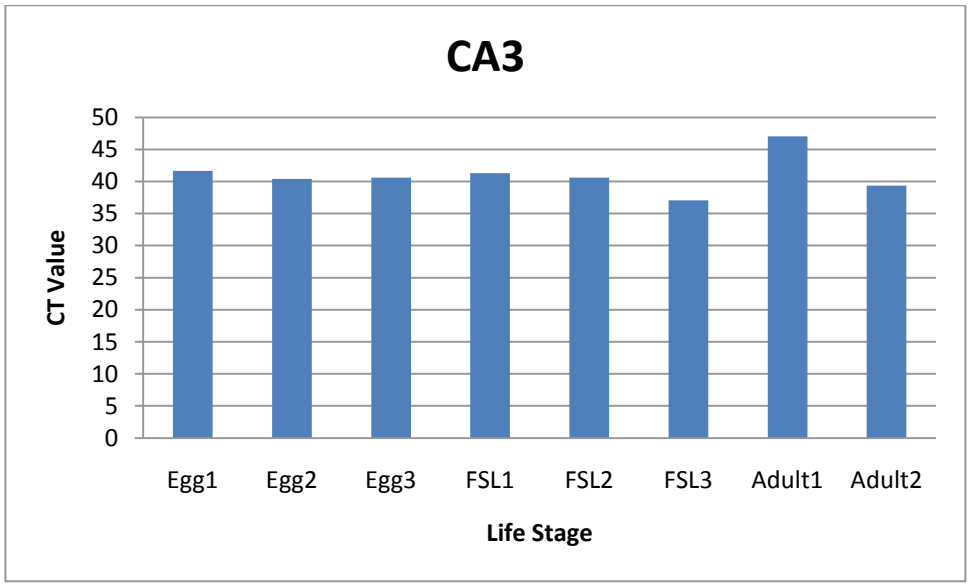

Figure 4.12 Gene expression of CA3 gene in egg, free swimming larvae and adult life stages of Ciona intestinalis.

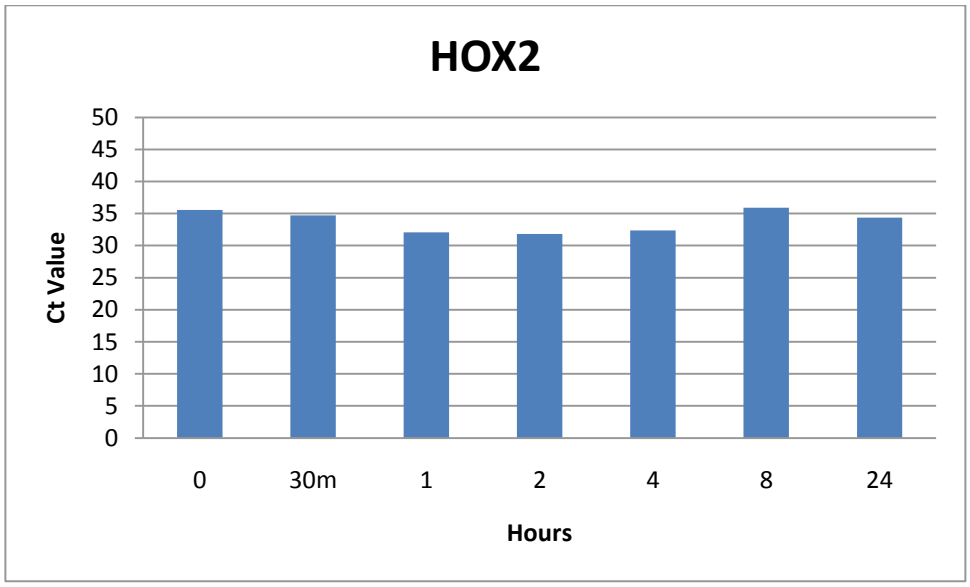

Figure 4.13 Transcript levels of HOX2 detected after 0-24 hours post mortem in Ciona intestinalis free swimming larvae.

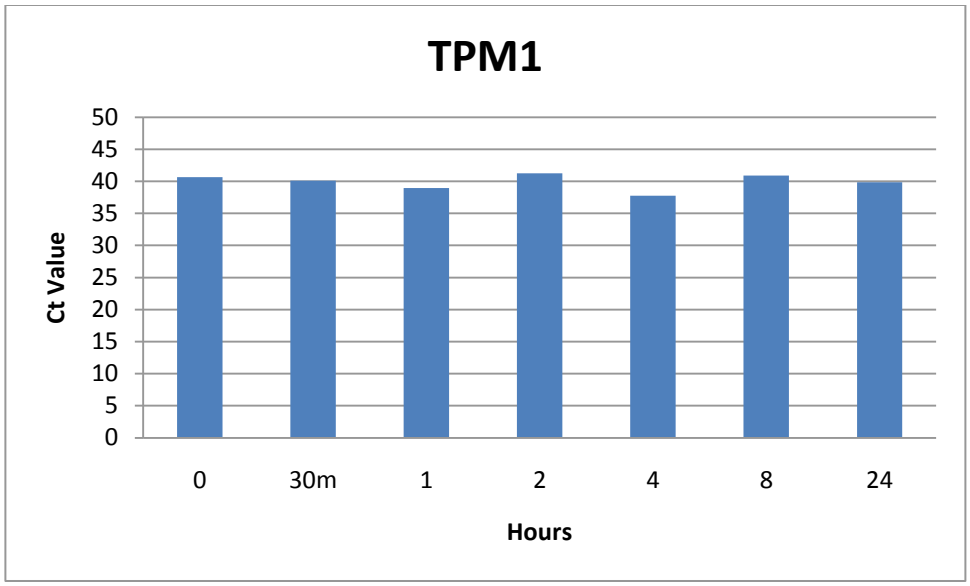

Figure 4.14 Transcript levels of TPM1detected after 0-24 hours post mortem in Ciona intestinalis free swimming larvae.

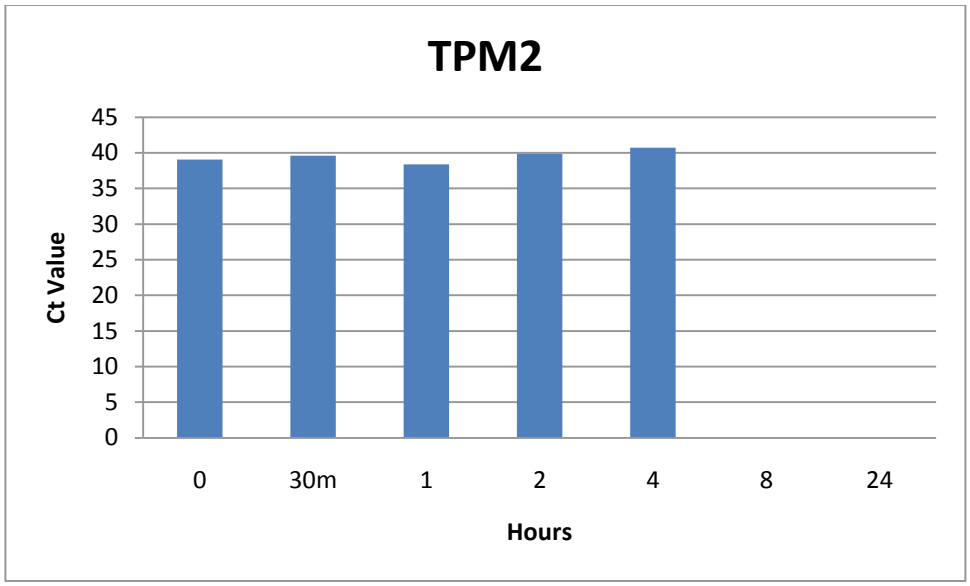

Figure 4.15 Transcript levels of TPM2 detected after 0-24 hours post mortem in Ciona intestinalis free swimming larvae.

4.4 Discussion

4.4.1. Normalisation Gene Analysis

As recommended by Bustin et al. (2005) we screened multiple normalisation genes and validated two that exhibited stable gene expression across multiple life stages in Ciona intestinalis: RPS27A and RPL11. These two genes were also validated as appropriate normalisation genes in a previous study involving Ciona intestinalis (Olinski et al. 2006). However four other normalisation genes that were used in other studies involving Ciona intestinalis, RPS18 (Olinski et al 2006), GAPDH (Olinski et al. 2006; Coric et al. 2008) calmodulin (CiCAM) (Piscopo et al. 2000; Matias et al. 2005) and α-tubulin (DeLigio & Ellington 2006), were too variable to be suitable as normalisation genes in this study. These findings underscore the importance of screening normalisation genes within each experimental situation instead of assuming that normalisation genes deemed appropriate in one study can be used universally in others. For this study, our normalisation genes will be used as positive controls instead of normalisation genes, since we are screening life stage genes for presence/absence of gene expression and not comparing gene expression levels across different samples.

4.4.2 Life Stage Analysis

Free swimming larvae

The temporal specificity of the TPM2 gene, as shown through the qRT-PCR results, makes it an excellent candidate as a marker for the free swimming larvae life stage of Ciona intestinalis. The free swimming larva is the only motile life stage of Ciona intestinalis and as such contains specific isoforms of muscle that are present in the larval tail but which are

lost during metamorphosis. The muscles that make up the adult heart and body wall consist of different isoforms of muscle protein (Chiba et al. 2003). EST studies have shown that TPM2 is one muscle gene that has shown to be exclusively expressed from the cleaving embryo to the larval life stage (Satoh et al. 2000; Chiba et al. 2003). These qRT-PCR results confirm this temporal specificity with expression detected in all free swimming larvae samples but not in any adult specimens. There was one egg sample that exhibited TPM2 expression; however the other pools of eggs did not making contamination a possible source for this unexpected expression of the TPM2 gene. By having a qRT-PCR assay that can distinguish free swimming larvae from other life stages of Ciona intestinalis is critical for aquaculture industry managers to determine when tunicates are reproductively active and thus posing a risk to aquaculture gear and mussel lines. Two other genes, HOX2 and TPM1 are also potential markers to distinguish between egg and free swimming larva life stages in water samples since our qRT-PCR results have shown that these transcripts are not present in egg samples but were always detected in free swimming larvae samples. This temporal specificity was also exhibited in fluorescence in situ hybridization (FISH) and whole-mount in situ hybridization (WMISH) experiments spanning egg to juvenile life stages, in which Ci-HOX2 was only detected during the larval like stage within trunk cells (Ikuta et al. 2004). In this study, these transcripts were also present in adult samples. However, Ciona intestinalis adults are sessile and remain attached to hard substrates for the remainder for their life cycle. As such, they are not normally present in the water column where sampling for eggs and larvae occur.

Unfertilized Egg

All of the genes targeted in this study as egg specific markers showed gene expression in all three life stages. In previous studies, no expression of ZF054 and ZF0364 were detected beyond the fertilized eggs stage with expression signals disappearing before the 16 cell stage (Miwata et al. 2006). Yamada et al. (2005) found PEM and PEM13 gene expression in the fertilized egg and the 8 cell stage embryo, however zygotic expression of this transcript was not detected (Yamada 2006). Other studies have shown that qRT-PCR is more sensitive than WMISH in detecting mRNA transcripts (Meyers-Wallen 2003; Thermes et al. 2006) which may explain the incongruence between our results and those of previous studies based on WMISH data. The present study was unable to find an unfertilized egg specific life stage marker for Ciona intestinalis. This life stage was particularly difficult to find potential target genes to screen for since so many of the transcripts present in eggs are maternally transcribed and therefore present in adults as well as egg life stages (Azumi et al. 2007). Nomura et al. (2009) conducted a proteomic analysis of three life stages of Ciona intestinalis (unfertilized egg, 16-cell embryo, tadpole larvae) in which they discovered 5 proteins that were uniquely expressed in the unfertilized egg. Two of these proteins could not be classified while the three others were closely correlated to proteins found in other organisms including: Glutamic-oxaloacetic transaminase 2b (GOT2), ATPase family, AAA domain containing 3A (ATAD3A), Isovaleryl Coenzyme A dehydrogenase (Nomura et al. 2009). Although Nomura et al. (2009) did not evaluate the expression of these three proteins in adults EST analysis has shown GOT2 gene expression in eggs, embryos, juveniles and young adults. Isovaleryl Coenzyme A dehydrogenase was also found in embryos and adults of Ciona intestinalis in EST analysis (Satou et al. 2004) and ATAD3A was found in eggs, embryos and adults (Satoh et al. 2000).

4.4.3 Transcript Stability

The quick post mortem degradation rate of the TPM2 transcripts evaluated in this study (4-8 hours) makes this gene an excellent indicator of viable Ciona intestinalis larvae. This is an important characteristic of the assay as non-viable larvae will not cause false positives if present in water samples. This is particularly important in screening mussel processing plant effluent water where water treatments are implemented to ensure that eggs and larvae of invasive tunicates are killed prior to exiting the plant into adjacent bays. These assays could be used to ensure that the treatment programs are functioning properly and that no viable larvae are being introduced to bays from effluent outflow. This viability assay could also be used to screen ballast water since non-viable larvae pose less of a risk to new regions than viable larvae. One other study (Hellyer et al. 1999) previously developed qRT-PCR assays to evaluate viability in Mycobacterium tuberculosis in order to rapidly evaluate drug susceptibility of specific strains.

4.4.4 Conclusion

Life stage specific assays based on qRT-PCR primers specific to the free swimming larvae life stage can be used to distinguish between egg and larvae of Ciona intestinalis in water samples. Such assays would be extremely useful in invasive species surveillance and monitoring programs. To distinguish between eggs, viable larvae and non-viable larvae in water samples, multiple markers could be used in conjunction with each other. The CIONINTESTCOI assay developed in chapter 2 could first be used to determine that Ciona intestinalis material is present in the water samples. The HOX2 or TPM1 markers

could then be used in a qRT-PCR assay to determine whether the Ciona intestinalis material is egg or larvae. Finally the TPM2 marker could then be used to evaluate whether the larvae are viable or non-viable. Such an assay would be beneficial in screening water samples around mussel processing plant effluent outflow as well as ballast water. While DNA based assays cannot distinguish viable material from non-viable, life stage specific qRT-PCR assays using transcripts with varying rates of transcript stability can be used to distinguish between viable and non-viable larvae.

It is important to note that these qRT-PCR assays have only been tested in laboratory conditions in artificial filtered seawater. Before being implemented into any surveillance or monitoring program, the assays would need to be validated in field conditions since contaminants and inhibitory compounds found in environmental water samples can impact assay sensitivity and efficacy (Tebbe & Vahjen 1993; Johnson et al. 1995; Wilson 1997; Toze 1999; Cunningham 2002).

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Chapter 5: Phylogeography of Botryllus schlosseri populations in Prince Edward Island, Canada

5.1 Introduction

The Golden Star tunicate, (Botryllus schlosseri) was first reported in Prince Edward Island (PEI) in 2001 in St. Peter's Bay (Locke et al. 2009). Since this initial detection on PEI, B. schlosseri has invaded 13 other areas of Prince Edward Island including: Savage Harbour, Tracadie Bay, South Lake, Souris Harbour, Boughton River, St. Mary's Bay, Murray River, Orwell River, Charlottetown Harbour, Port Borden, Summerside Harbour, West Point and Alberton Harbour (Art Smith, Aquatic Invasive Species Coordinator, Department of Fisheries and Oceans, Canada, personal communication). This species is currently causing significant fouling problems to shellfish aquaculture in Prince Edward Island by fouling mussel socks, buoys and aquaculture gear.

B. schlosseri has been described as a cosmopolitan species, with populations in every continent except Antarctica (Van Name 1945; Yund & O'Neil 2000). It is currently found on both Atlantic and Pacific coasts of Canada and the United States, although it is not an indigenous species to North America (Lambert & Lambert 1998; Stoner et al. 2002; Dijkstra et al. 2007). Some believe that B. schlosseri originated in the Mediterranean (Berrill 1950) although Carlton (2005) speculates that this tunicate may have originated in the Pacific Ocean since botryllid diversity is highest in the Pacific Ocean. B. schlosseri likely spread from its native population to non-native sites via shipping (Lambert & Lambert 1998; Stoner et al. 2002; Lopez-Legentil et al 2006; Dijikstra et al 2007).

The source population for the initial incursion of Golden Star to Prince Edward Island is unknown, as is(are) the vector(s) that brought Golden Star to this region. There have been two main suggested vectors for the Golden Star incursion to Prince Edward Island. Golden Star samples were found and collected from a barge that was present in Savage Harbour, PEI in 2002 (Locke et al. 2009). However, the Golden Star tunicate was already present in the adjacent St. Peter's Bay in 2001, prior to the barges presence along the north shore of PEI. It is unknown whether this barge was the source of the 2002 invasion of Golden Star in Savage Harbour in 2002, or if Golden Star spread to Savage Harbour from the adjacent St. Peter's Bay. The second suggested vector for the Golden Star invasion in PEI has been the transfer of shellfish between Nova Scotia and eastern PEI. Regular transfers of oysters occurred prior to the arrival of Golden Star between Eastern PEI and the Bras d'Or Lakes (Locke et al. 2007). There are also many other possible vectors which may have brought Golden Star to PEI including recreational boat traffic and ballast water transfer. No study has yet to use molecular tools to examine the source populations for the PEI invasion. Possible vectors could be identified by determining possible source populations for this invasion to PEI. This information could be then used to minimize the risk of the same vector transporting more invasive species to this region.

In this study, both 18S rDNA and cytochrome oxidase I (COI) gene sequences were examined from Golden Star populations on Prince Edward Island, and compared to those of Botryllus schlosseri samples collected elsewhere in Canada (including from the barge which may be a possible vector), US and Europe, as well as to sequences from other studies including Mediterranean, European Atlantic and US Atlantic populations (Lopez-Legentil et al. 2006), and Japan (Yokobori et al. 2006). The 18S rDNA gene has been used

in many studies to clarify taxonomic relationships amongst tunicates, as this nuclear gene is highly conserved within species (Wada et al. 1992; Wada 1998; Swalla et al. 2000; Stach & Turbeville 2002; Yokobori et al. 2006; Zeng et al. 2006). The 18S rDNA gene is often too conserved to yield intraspecies level taxonomic information so was used to confirm species identification in this study. Mitochondrial genes such as COI have proved useful in elucidating taxonomic relationships at the species and intraspecies level, since mitochondrial genes typically evolve at a faster rate than nuclear genes (Stach & Turbeville 2002; Turon & Lopez-Legentil 2004; Lopez-Legentil et al. 2006). For this reason, the COI gene was used in this study to compare intraspecies variation between populations of Botryllus schlosseri.

The objective of this study was to use phylogenetic analysis to identify possible source populations of the invasion of Golden Star to Prince Edward Island as well as to evaluate how this species spread across PEI after the initial invasion in St. Peter's Bay. It is critical to understand how invasions spread at the local level so that future invasions can be prevented and/or better managed.

5.2 Materials and Methods

Botryllus schlosseri specimens were collected from 10 locations surrounding Prince Edward Island, Canada (St. Peter's Bay, Orwell River, Savage Harbour, St. Mary's Bay, Tracadie Bay, Souris Harbour, Cardigan River, Nine Mile Creek, Murray River, Boughton River) three locations in North America (Magdalen Islands, Quebec, Canada; Ladysmith, British Columbia, Canada; Salem, Massachusetts, USA) two locations in Europe (Carlingford, Northern Ireland; Villagarcia de Arousa, Spain) and five locations in Japan

(Table 5.1). For all PEI populations, with the exception of Souris Harbour, Orwell River and Nine Mile Creek, >10 samples were collected from each geographic location with no two collected samples being closer than 1 metre from any other sample. Souris Harbour and Nine Mile Creek were newly invaded regions so in these cases samples were limited to 2 and 4 samples respectively due to small population sizes. All samples collected in this study were collected from artificial substrate with the exceptions of the 3 samples from Savage Harbour, PEI (which were collected from eelgrass beds) and 4 samples from Nine Mile Creek, PEI (which were collected from larvae in the water column). Source tissue from the barge samples were from paraffin blocks which had been embedded for histology in 2002. All DNA extractions were performed using QIAamp DNA MiniKits (Qiagen Inc, Canada) according to manufacturer's instructions for either tissue extractions or paraffin embedded tissue extractions.

Table 5.1 Samples sequenced in this study.

Species	Geographic Location	Coordinates	Number of Samples
Botryllus schlosseri	St. Peter's Bay, PEI Canada	46 25 28N 62 37 28W	12
Botryllus schlosseri	Tracadie Bay, PEI Canada	46 23 34N 62 59 34W	13
Botryllus schlosseri	Souris Harbour, PEI Canada	46 20 53N 62 15 00W	2
Botryllus schlosseri	Murray River, PEI Canada	46 1 42N 62 32 58W	15
Botryllus schlosseri	Savage Harbour, PEI Canada	46 24 57N 62 49 53W	18
Botryllus schlosseri	Orwell River, PEI Canada	46 08 56N 62 53 35W	1
Botryllus schlosseri	Boughton River, PEI Canada	46 15 54N 62 27 34W	10
Botryllus schlosseri	St. Mary's Bay, PEI Canada	46 7 38N 62 30 55W	10
Botryllus schlosseri	Nine Mile Creek PEI Canada	46 8 52N 63 13 3W	4
Botryllus schlosseri	Cardigan River PEI Canada	46 15 00N 62 41 00W	10
Botryllus schlosseri	Havre aux Maisons, Magdalen Islands QC, Canada	47 25 6N 61 49 2W	5
Botryllus schlosseri	Havre Aubert, Magdalen Islands QC Canada	47 12 56N 61 59 3W	5
Botryllus schlosseri	Ladysmith, BC, Canada	49 00 17N 123 48 57W	1
Botryllus schlosseri	Yarmouth, NS Canada	43 49 55N 66 7 27W	12
Botryllus schlosseri	Carlingford, Northern Ireland	54 3 46N 6 9 16	4
Botryllus schlosseri	Ria de Arousa, Galicia Spain	42 34 12 8 50 34	4
Botryllus schlosseri	Salem, MA, USA	42 31 12N 70 52 55W	1
Botryllus schlosseri	Otaru, Japan	43 11 8N 141 1 25E	2
Botryllus schlosseri	Sugashima, Japan	34 29 4N 136 52 33E	1
Botryllus schlosseri	Usujiri, Japan	41 56 9N 140 56 59E	1
Botryllus schlosseri	Misaki, Japan	35 00 56N 139 36 42E	1
Botryllus schlosseri	Akkeshi, Japan	43 01 16N 144 01 25E	1
Botrylloides violaceus	Washington, USA	48 32 21N 123 00 54W	1
Botrylloides violaceus	Ria de Arousa, Galicia, Spain	42 34 12 8 50 34	2
Botrylloides violaceus	Mount Dessert Island, MA USA	44 16 37N 68 19 19W	8
Botrylloides violaceus	Akkeshi Japan	43 01 16N 144 01 25E	1

Table 5.2 Sequences used in this study from GenBank.

Botryllus schlosseri isolate HS	COI	DQ340224
Botryllus schlosseri isolate HR	COI	DQ340223
Botryllus schlosseri isolate HQ	COI	DQ340222
Botryllus schlosseri isolate HV	COI	DQ340221
Botryllus schlosseri isolate HW	COI	DQ340220
Botryllus schlosseri isolate HU	COI	DQ340219
Botryllus schlosseri isolate HT	COI	DQ340218
Botryllus schlosseri isolate HP	COI	DQ340217
Botryllus schlosseri isolate HO	COI	DQ340216
Botryllus schlosseri isolate HN	COI	DQ340215
Botryllus schlosseri isolate HM	COI	DQ340214
Botryllus schlosseri isolate HL	COI	DQ340213
Botryllus schlosseri isolate HK	COI	DQ340212
Botryllus schlosseri isolate HJ	COI	DQ340211
Botryllus schlosseri isolate HI	COI	DQ340210
Botryllus schlosseri isolate HG	COI	DQ340208
Botryllus schlosseri isolate HH	COI	DQ340209
Botryllus schlosseri isolate HF	COI	DQ340207
Botryllus schlosseri isolate HE	COI	DQ340206
Botryllus schlosseri isolate HA	COI	DQ340205
Botryllus schlosseri	COI	AY600987
Botryllus tyreus	COI	DQ365851
Botryllus schlosseri	18S	AB211066
Botryllus schlosseri	18S	FM244858
Botryllus planus	18S	DQ346653

CASIS and CAS2 primers (LeRoux et al. 1999) were used in PCR to amplify a 522bp section of the 18S rDNA gene from one specimen from each locality to confirm species identification. BOTSCHLOCOI-F1 and R1 primers (Stewart-Clark et al. 2009) were used in PCR to amplify a 351bp section of the COI gene to evaluate haplotypes within populations.

PCR was performed in a 25µl final volume containing 12.5 µl AmpliTaq Gold PCR Master Mix (Applied Biosystems manufactured by Roche, Branchburg, New Jersey), 10 pmol appropriate forward and reverse primers, 25-60 ng DNA and 9.5 µl sterile ddH_20. Samples were denatured for 3 minutes at 92^oC, amplified over 35 cycles consisting of 1 minute at 94 ^oC for denaturation, 1 minute at 53 ^oC for primer annealing and 3 minutes at 72 ^oC for elongation. Following the last cycle, polymerization was extended for 5 minutes at 72^oC to complete elongation.

PCR amplicons were separated in 1% agarose gels containing 0.5µg ml^{-1} ethidium bromide and visualized using ultraviolet light. PCR amplicons were then sequenced in both directions by Laboratory Services-Molecular Biology Section, University of Guelph.

The identity of the generated COI and 18S rDNA sequences was confirmed by BLAST analysis (Altschul et al. 1997) of the GenBank database. The ClustalW application in MEGA (Larkin et al. 2007; Tamura et al. 2007) was used to perform multiple sequence alignments between the Botryllus schlosseri sequences generated in this study with all 18S

rDNA and COI botryllid sequences currently listed in GenBank (Table 5.2) in order to perform phylogenetic analyses.

Neighbour-joining (NJ), Kimura 2-parameter model with gaps and missing data handled by complete deletion and maximum parsimony (MP) phylogenies were constructed using both 18S rDNA and COI sequences with MEGA (Beta) version 4.1 (Tamura et al. 2007, Kumar et al. 2008). Statistical support for MP, and NJ tree topologies were bootstrap-resampled 1000 times (Felsenstein 1985). Ciona intestinalis was used as an outgroup for the 18S rDNA tree and Botryllus tyreus was used as an outgroup for the COI tree (Lopez-Legentil et al. 2006).

5.3 Results

5.3.1 18SrDNA

The 18S rDNA sequences generated from the Botryllus schlosseri specimens used in this study from North American populations were identical to each other when aligned. All of the 18S rDNA sequences generated from North American B. schlosseri samples (from both Pacific and Atlantic coasts) grouped with each other during phylogenetic analysis (Figure 5.1). In addition, these North American B. schlosseri sequences aligned with sequences generated from two populations in Japan (Sugashima and Otaru). The 18S rDNA sequence from Sugashima was identical to the North American sequences while the sequence from Otaru differed by only one transition. The 18S rDNA sequences generated from European Atlantic coast populations of B. schlosseri (from Northern Ireland and Spain) aligned with each other in a separate group than the North American sequences (Figure 5.1). Existing 18S rDNA sequences for B. schlosseri obtained from GenBank did not align with either of

these two groups of sequences generated in our study. No B. schlosseri 18S rDNA sequence was generated from the paraffin embedded block sample of the barge samples.

5.3.2 COI

All of the PEI Golden Star COI sequences generated from samples collected from artificial substrate in this study were identical to each other and to B. schlosseri isolate HS as well as to the haplotype from Nova Scotia (GenBank Accession #DQ340224). The B. schlosseri sequence from British Columbia, Canada differed from the PEI sequences by two transitions. The B. schlosseri sequence from Northern Ireland differed from the PEI sequences by 10 transitions and the B. schlosseri sequences from Villagarcia de Arousa, Spain differed from the PEI sequences by 17 transversions and 33 transitions. The Magdalen Island population of B. schlosseri consisted of three COI haplotypes, one which was identical to the PEI haplotype.

When the COI sequences generated in this study from Botryllus schlosseri were aligned with the sequences of Lopez-Legentil et al (2006), all PEI sequences collected from specimens growing on artificial substrate grouped into the same clade (Figure 5.2). These sequences from PEI, NS and one from Salem MA, USA grouped most closely with the haplotype HR and HS from Wood's Hole MA, USA. Of the PEI samples collected from native substrate, one sample differed from all other PEI sequences by 7 transitions. This second haplotype from PEI grouped most closely with haplotypes from the Magdalen Islands, France and Maine, US. The sample from British Columbia, Canada grouped most closely with the haplotype from Otaru, Japan. The Magadalen Islands haplotype 1 (H1) grouped most closely with the predominant PEI haplotype. Magdalen Islands haplotype 2

(H2) grouped most closely with haplotypes from six harbours in the Mediterranean and European harbours. Magdalen Islands haplotype 3 (H3) grouped most closely with haplotypes from Maine and France and the second haplotype from PEI.

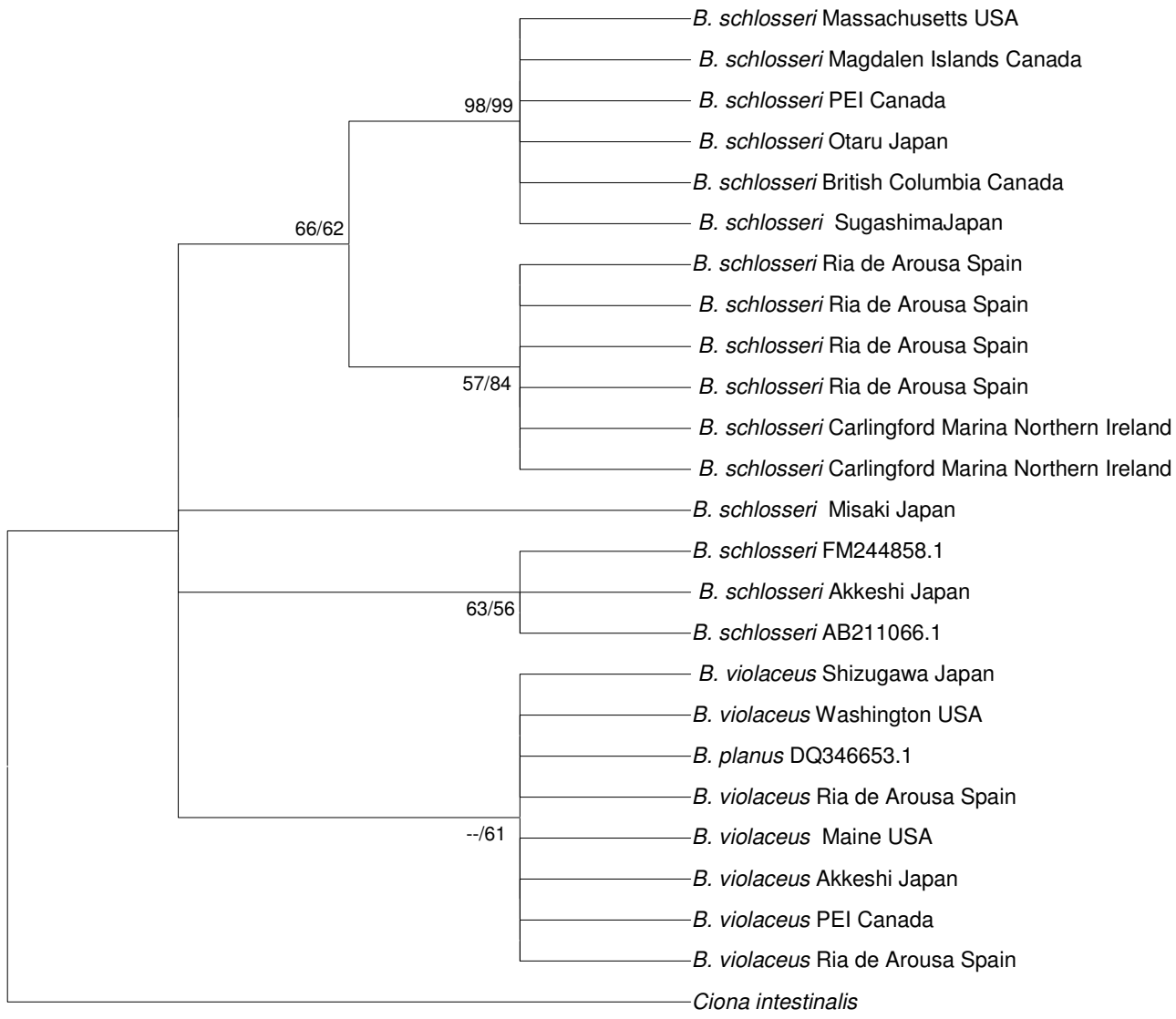

Figure 5.1 Phylogenetic tree for ***Botryllus schlosseri*** based on 18S rDNA sequences from this study and other botryllid sequences from GenBank. The Neighbour-joining tree with *Ciona intestinalis* as the outgroup is shown. Values at nodes represent the bootstrap percentages from 1000 replicates for maximum parsimony and neighbour-joining respectively.

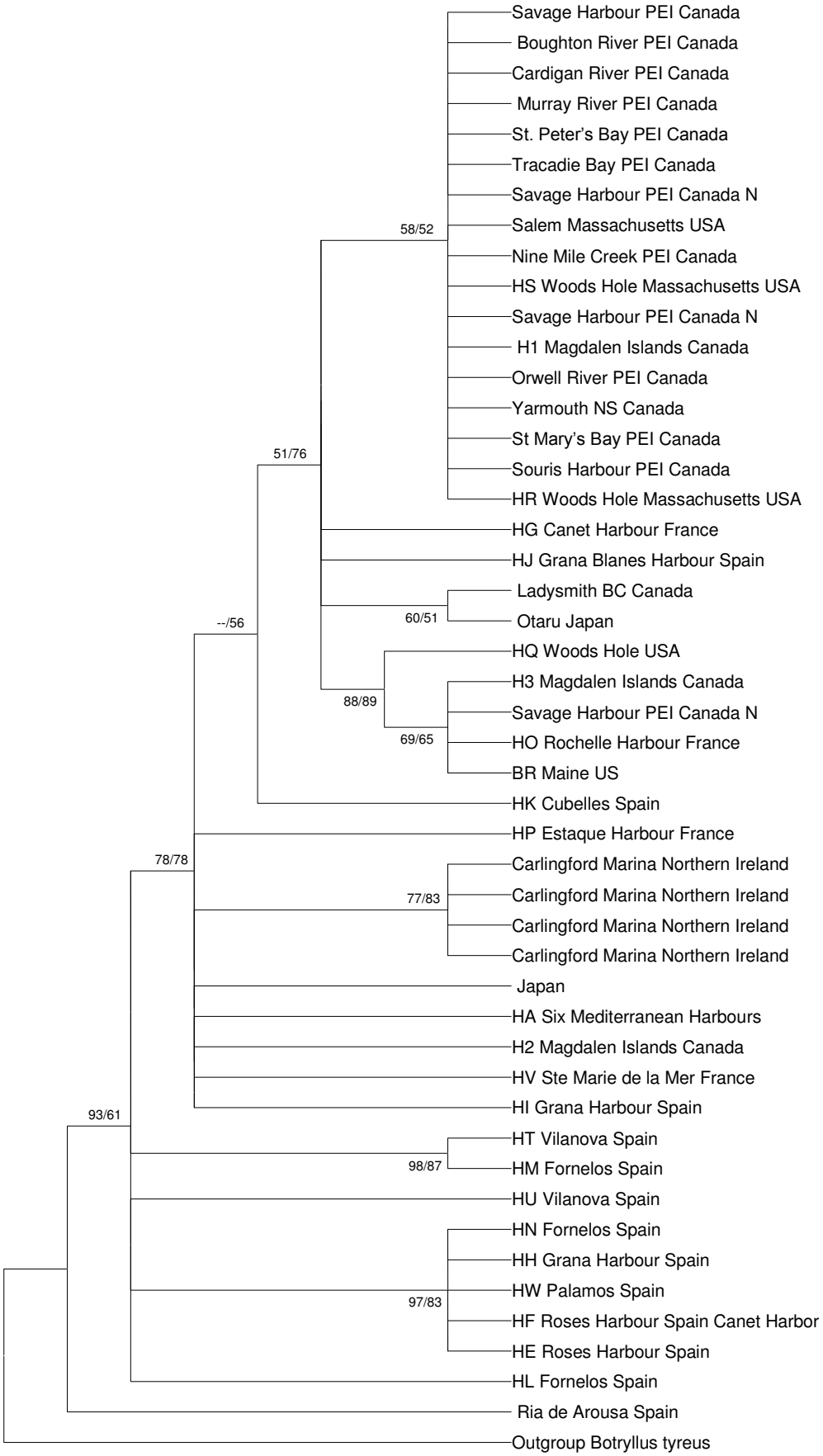

Figure 5.2 . Phylogenetic tree for *Botryllus schlosseri* based on COI sequences from this study and sequences from Lopez-Legentil 2006. The neighbour-joining tree with *Botryllus tyreus* as the outgroup is shown. Values at nodes represent the bootstrap percentages from 1000 replicates for maximum parsimony and neighbour-joining respectively.

5.4 Discussion

5.4.1 18S rDNA

There has been much discussion on whether Botryllus schlosseri is a cosmopolitan species, or in fact a group of different species. Several studies have highlighted the polymorphic differences in B. schlosseri populations from different locations. Stoner et al. (2002) determined that the US Atlantic and Pacific coast populations are so genetically different, they are either two different species, or they were founded by two genetically distinct populations of the same species (Stoner et al. 2002). Boyd et al. (1990) also speculate that Botryllus schlosseri might be more than one species. These authors base their assumption on the variation seen in morphological characteristics including: 1) egg size 2) protostigmata number in oozooids 3) pallial bud number in first blastogenic generation 4) number of stigmatal rows and 5) haploid chromosome number (Van Name 1945; Berrill 1950; Boyd et al. 1990). While some allorecognition studies (Boyd et al. 1990; Rinkevich et al. 1992) found that Atlantic US and Pacific US Botryllus schlosseri colonies did not fuse with each other, other studies including (Rinkevich et al. 1992) found that colonies in Pacific USA and Japan did fuse with each other.

The 18S rDNA phylogenetic analysis separated the North American/Japanese Botryllus schlosseri populations from the two populations that we sampled in Europe (Spain and Northern Ireland). In addition, two existing B. schlosseri sequences from GenBank (AB211066.1 and FM248858) and a sequence from Akkeshi Japan from this study grouped together in a separate group. This level of separation between B. schlosseri specimens based on 18S rDNA phylogenetic analysis was unexpected since the 18S gene is usually quite conserved at the species level. This genetic variation may be preliminary evidence

that the current organisms that are being identified as B. schlosseri may in fact be more than one species. However, more sampling from more geographic locations would be necessary to confirm such a hypothesis. Based on 18S rDNA sequence analysis it does appear that the PEI invasion of B. schlosseri occurred from another North American population or from Japan and not from Europe.

All of the Botrylloides violaceus sequences in this study grouped separately to the Botryllus schlosseri sequences, indicating that this gene section is variable enough to distinguish between different botryllid speices. A Botryllus planus sequence from GenBank (DQ346653) grouped together with the Botrylloides violaceus sequences. The 18S sequence of this B. planus sequence is identical to the B. violaceus 18S sequences, indicating that the B. planus sequence may have been generated from a misidentified B. violaceus specimen. Botryllids are difficult to distinguish from one another and the occurrence of different colour morphs of each species further complicates species identifications. Gretchen Lambert (personal communication) uses position of ovary and testes to distinguish between Botrylloides sp. and Botryllus sp. however such characterization requires careful dissection that does not always occur in field surveys. Carver et al. (2006) also indicate that B. schlosseri and B. violaceus are often confused with other botryllids including B. leachii. A comprehensive study consisting of multiple samples from multiple geographic locations of multiple botryllid species would be needed to compare both molecular and morphological characteristics to clarify the taxonomy of the botryllid group of tunicates.

5.4.2.COI

Recent molecular studies involving the COI gene have indicated that the Botryllus schlosseri populations in the Mediterranean, US Atlantic Coast and European Atlantic Coast are all populations of the same species (Lopez-Legentil et al 2006). The present results with the COI gene are consistent with this theory. However, like the 18S rDNA analysis the COI phylogenetic analysis also separates the North American haplotypes (in addition to one sample from Japan and two from Europe (France and Spain) from all other Botryllus schlosseri haplotypes. Only one haplotype from North America (H2 from the Magdalen Islands) groups outside of the predominant North American grouping.

Based on the COI sequences in this study, it appears that the Golden Star populations on PEI are most closely related to the Golden Star populations in Yarmouth Nova Scotia, Magdalen Islands, Wood's Hole and Salem, Massachusetts, indicating that the Massachusetts, Nova Scotia and Magdalen Island populations are possible source population for the PEI invasion. Although there is much shipping traffic between Massachusetts and Atlantic Canada, there are very few direct shipping routes from Massachusetts to Prince Edward Island (DFO 2006). Most of the shipping traffic that arrives in Atlantic Canada from Massachusetts arrives by way of Nova Scotia (DFO 2006). It is therefore possible that the Golden Star tunicate moved from Massachusetts to Nova Scotia via shipping traffic, and another vector moved this tunicate from Nova Scotia to Prince Edward Island. One possible vector that may be responsible for the movement of Golden Star from NS to PEI is oyster transfers, which occurred regularly between the Bras D'Ors Lakes, NS to Eastern Prince Edward Island (Locke et al. 2007). Recreational or

fishing boats are also possible vectors for the NS to PEI invasion since there is considerable small craft movement between Nova Scotia and Prince Edward Island.

Until 2009, only one COI haplotype of Botryllus schlosseri was detected in all populations in Prince Edward Island. This COI data suggests that one initial invasion brought very small colonies of this species to PEI, and local activity spread this species from bay to bay. This confirms with molecular data, the suggestion by Locke et al. (2007) that invasive tunicates in PEI likely spread by local boating activity after their initial invasion. The present data also suggests that the Savage Harbour invasion did spread from the initial St. Peter's Bay population since these two populations have identical haplotypes. However, since we were unable to generate sequences from the Botryllus schlosseri samples collected from the barge, we can only speculate that it may be possible that the samples from the barge consisted of that same haplotype as well and therefore did contribute to a secondary invasion.

The second COI haplotype of Botryllus schlosseri was detected in Savage Harbour in 2009 after Botryllus schlosseri populations growing on natural substrates were sampled. B. schlosseri is found predominantly on artificial substrate on PEI (including wharves, bridge pilings, buoys, boat hulls and aquaculture lines and gear) as PEI bays have sandy bottoms with very little rocky bottom habitats available for B. schlosseri on natural substrate. As a result, all of our sampling in 2008 was from populations growing on artificial substrate. However after the initial sampling for this study was completed and only one haplotype was detected, we were concerned that this sampling bias towards artificial substrate may not reflect the true haplotype diversity of this species on PEI. There remained the

possibility that this invasive haplotype preferred artificial substrate and that other haplotypes may be present that preferentially grow on natural substrate. As a result, samples from natural substrates were sampled in 2009, during which the first haplotype was detected, along with a second haplotype. There are two possibilities that can explain the discovery of this second haplotype of Botryllus schlosseri on PEI. One is that this haplotype does prefer growing on natural substrate and thus has not been detected on any of the previous sampling from artificial substrates. The second possibility is that this second haplotype was discovered in 2009, and all other sampling for this study occurred during 2008. A new invasion of Botryllus schlosseri containing this haplotype may have occurred between these two sampling time periods. This second COI haplotype detected on PEI groups most closely with haplotype 3 from the Magdalen Islands, from haplotype BR from Maine and haplotype HO from France. There is a ferry that runs between Souris PEI and the Magdalen Islands and it is possible that this ferry is a vector for this species between the two regions.

The COI sequence of the H2 Magdalen Islands Botryllus schlosseri population was more similar to European Atlantic Ocean and Mediterranean Ocean populations than other North American Atlantic Coast populations. This indicates that the source of these Magdalen Islands population is most likely Europe or the Mediterranean and separate from the PEI invasion.

The present findings highlight the impact that recreational boaters and local fisheries and aquaculture can have in spreading an invasive species once it has been brought to a new region. It is clear that educational programs at the local level are required to help minimize

the local transfer of invasive species after they have been transferred to a new region. Once a new invasive tunicate is introduced to PEI waters, it has historically been difficult to contain in the initial region of the invasion. Therefore, minimizing the risks of the initial invasion may be the best defence that this region has against future invasive tunicate species. This study also highlights the importance of regularly monitoring haplotypes in invasive populations to evaluate whether vectors continue to transport more organisms of an invasive species to the invaded region.

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Chapter 6: Discussion and Conclusions

6.1 Species Specific Assays

The species specific invasive tunicate assays developed in this study were screened at multiple stages to ensure that the final assays were as specific, sensitive and effective as possible. Primers were first screened using computer software to ensure that secondary structures, heterodimers and homodimers would not impact assay efficacy and that primer regions were unique to each species. Primers were then evaluated in laboratory controlled conditions for sensitivity and specificity before moving onto field trial testing. Within field trials, the assays were tested in multiple water sample types, using two different sampling methods, and two different DNA extraction methods. As a result of these three stages of testing (computer software, laboratory and field) the COI and 18S rDNA primers that were developed in this study are highly sensitive, specific and efficacious in detecting microscopic levels of invasive tunicate eggs and larvae in water samples.

In laboratory conditions, these four assays were highly sensitive detecting down to 1 egg, larvae or zooid in water samples. These assays were successful in detecting tunicate samples from many geographic locations, including multiple locations in Canada, United States, Spain, Ireland, Northern Ireland and Japan. This indicates that the gene regions from which the primers were developed are conserved enough to detect these four species from different regions. In addition, the colonial assays were tested using different colour morphs of each species to ensure that the assays could detect all colour morphs as the same species. The four species specific assays also exhibited 100% specificity when tested against the other invasive tunicates present on Prince Edward Island as well as a native

tunicate Molgula sp. and an invasive tunicate (Didemnum vexillum) which is considered a high risk as a "future" invasive species in this region (Locke 2009). After laboratory testing was completed, it was evident that the assays were ready for field validation experiments.

During field testing, the sensitivity and repeatability of the four assays were tested in two different water sample types (mussel processing plant effluent and bay water surrounding mussel leases), using two different water sampling methods (direct grab and sieve-concentrated), and two different DNA extraction methods (Qiagen DNeasy and Norgen Soil DNA Isolation kits). Water sampling type and the DNA extraction method had a significant impact on assay efficacy in this study which highlights the importance of validating assays in the specific conditions of usage. Although few PCR based studies include the impact of DNA extraction methods on assay efficacy, Darling and Blum (2007) highlight this step as one of the most critical steps in PCR based assays. This was certainly the case in the present study. Although both DNA extraction methods produced comparable quantities of DNA, there was a clear difference during PCR with respect to the level of PCR inhibitors present in each DNA sample. The mussel processing plant effluent water was higher in sediment than the bay water surrounding mussel leases making it higher in PCR inhibitors than the bay water samples. The Qiagen DNeasy extraction kit was not able to separate the inhibitors from the DNA samples and as a result the four species specific assays did not perform well with DNA extracted from Qiagen DNeasy kits. However, when the Norgen Soil DNA Isolation kit was used to extract DNA from mussel processing plant effluent water samples, there were no issues with PCR inhibition and assay sensitivity was excellent detecting as few as 1-5 eggs and larvae in water

samples. Conversely, in bay water samples, the DNA samples extracted using the Qiagen DNeasy kit were amplifiable during PCR whereas the Norgen extracted DNA samples were not. The mechanical vortexing, which is a component of the Norgen extraction method, shears the DNA in samples without sediment making the DNA unamplifiable by PCR. Assay sensitivity and repeatability in Norgen extracted mussel processing effluent were also increased when DNA samples were diluted 10-fold prior to the PCR reaction. These results show the impact that PCR inhibitors can have on assay sensitivity and efficacy and that assays must be validated when used indifferent water conditions. If appropriate DNA extraction methods and PCR protocols are not followed, false negatives may have a large impact on assay efficacy.

The end result of the field testing experiments in this study yielded two different protocols for these assays in each of the two different field water sample types: mussel processing effluent and bay water. Following these two protocols the four species specific assays showed high sensitivity and repeatability in detecting small quantities of eggs, larvae and zooids of C. intestinalis, S. clava, B. violaceus and B. schlosseri in both water sample types. These assays also performed similarly to the current standard method of light microscopy for detecting egg and larval stages of invasive tunicates in water samples in PEI (Gill et al. 2007). However, the molecular assays can screen water samples in a more high-throughput manner, especially now with the advent of 96 well plate thermocyclers and technologies incorporating capillary electrophoresis (e.g. Qiagen QIAxcel) that replace traditional horizontal agarose gel electrophoresis are automating the PCR process.

As aquatic invasive species spread around the world, governments at all levels are launching rapid response protocols to ensure that logistical decisions, protocols and communication plans are in place prior to the arrival of aquatic invasive species so that any such invasion is appropriately handled to minimize risk to the newly invaded area. A key component in each of these rapid response protocols is the development and implementation of early detection procedures so that invasive organisms can be detected prior to their population levels spiking to sizes that make eradication or control difficult or impossible (National Invasive Species Council 2003; Canadian Council of Fisheries and Aquaculture Ministers Aquatic Invasive Species Task Group 2004; Washington State Aquatic Nuisance Species Committee 2005; MAF Biosecurity New Zealand 2007). This study has shown that COI and 18S rDNA gene markers can be used in monitoring programs to detect invasive tunicate eggs and larvae in environmental water samples surrounding PEI. Based on species distribution and vector pathways in Atlantic Canada, Locke (2009) has created a list of the 17 most likely tunicate species to next invade Atlantic Canada. One of the tunicates on this list, Diplosoma listerianum, has recently been detected in the Gulf of St. Lawrence region in the Magdalen Islands in 2008. Species specific COI primers have also been developed for use in PEI to monitor for this invasive tunicate (Willis & Stewart-Clark unpublished data). Future studies could involve the development of molecular assays to detect these 16 additional tunicate species so that monitoring programs can be implemented for their early detection.

Due to the fact that the four assays in this study were designed to monitor mussel effluent and mussel lease water for known tunicate species, the assays were designed to be very specific at the species level. Molecular assays designed to screen water samples for the

presence of these "future" invasive tunicates could be designed to be less specific to increase the scope of their detection capabilities. For example genus specific primers could be designed for invasive tunicates, and if a positive result is generated, RFLP or nested primers could then be used to increase the assay specificity to the species level.
One of the clear benefits of PCR based detection is that thermocyclers and gel electrophoresis are now components of every molecular biology laboratory. As a result assays with clearly validated protocols can be transferred to other laboratories so that once developed, assays are available to any jurisdiction.

6.2 Life stage and Viability Assay

qRT-PCR assays were designed in this study to detect and distinguish the free swimming larvae life stage of C. intestinalis from other life stages of this invasive tunicate. To act as positive controls in this assay across all life stages RPS27A and RPL11 genes were validated as stable normalisation genes (as in Olinski et al. 2006). Four other normalisation genes that were used in other studies involving Ciona intestinalis, RPS18 (Olinski et al. 2006), GAPDH (Olinski et al. 2006; Coric et al. 2008) calmodulin (CiCAM) (Piscopo et al. 2000; Matias et al. 2005) and α-tubulin (DeLigio & Ellington 2006), were too variable to be suitable as normalisation genes in the present study. These results support the recommendations by Bustin et al, (2005) that normalisation genes must be validated within each experimental situation instead of assuming that normalisation genes deemed appropriate in one study can be used in others.

TPM2 gene expression showed temporal specificity in the free swimming larvae life stage of C. intestinalis making this gene an ideal marker for this life stage. In addition, HOX2

and TPM1 were expressed in free swimming larvae and adult life stages but not in egg samples of C. intestinalis. These two genes also could be potential markers for the free swimming larvae life stage in ballast water or surface water samples since adult life stages are sessile and typically not present in the water column. However, fragments of adult Ciona intestinalis may be present in mussel processing effluent since tunicates are fragmented while being removed from the mussel socks during cleaning in the processing facility. A free swimming larvae detection assay would be useful to industry as it would delineate for mussel growers when Ciona intestinalis adults were successfully reproducing in the area of their mussel lease which impacts when treatments are applied on their mussel lines to control Ciona intestinalis fouling.

In addition to detecting whether the free swimming larvae life stage of Ciona intestinalis is present in a water sample, the fast post-mortem degradation of the TPM2 transcript (4-8 hours) also makes it an ideal assay to determine viability of the Ciona intestinalis larvae. Due to the fast degradation rate, the TPM2 assay will detect live C. intestinalis larvae and not larvae that have died (within 4-8 hours). This viability screening is critical in mussel processing effluent water since only the release of live larvae in mussel processing plant effluent pose a risk to adjacent bays. This viability assay could also be useful in ballast water testing since viable larvae pose a higher risk to new regions than dead larvae (which would be detected by DNA based assays). It is important to note that these assays have only been tested in controlled laboratory conditions. The assay would have to be validated in field conditions prior to implementation in any surveillance or monitoring program. As noted and explored with the species specific assays, PCR inhibitors present in environmental samples can impact assay efficacy and sensitivity and will require further

investigation for the life stage and viability assay (Tebbe & Vahjen 1993; Johnson et al. 1995; Wilson 1997; Toze 1999; Cunningham 2002).

Additional qRT-PCR studies could be performed to make the life stage and viability assay quantitative. mRNA from known quantities of Ciona intestinalis larvae (1,5,10,20,50,100 etc) could be used in qRT-PCR to create a standard curve of TPM2 gene expressions. Samples containing unknown quantities of larvae could then be evaluated using this standard curve to quantify the number of larvae present in water samples. Such an assay would be useful in measuring propagule pressure from ballast water or mussel effluent waste water, or in evaluating the level of infestation in bays surrounding mussel leases.

6.3 Phylogeography of Botryllus schlosseri populations in Prince Edward Island

There has been much discussion on whether Botryllus schlosseri is a cosmopolitan species, a group of different species, or whether differences between populations of B. schlosseri are due to misidentifications with other botryllid species (Boyd et al. 1990; Stoner et al. 2002; Carver et al. 2006). In the present study a higher level of variability was found in the 18S rDNA gene of species identified as Botryllus schlosseri than was expected. The 18S rDNA phylogenetic analysis separated the North American/Japanese Botryllus schlosseri populations from the two populations sampled from Spain and Northern Ireland. In addition, existing B. schlosseri sequences from GenBank (AB211066.1 and FM244858) and a sequence generated from a B. schlosseri specimen from Akkeshi Japan from this study grouped together but separately from the other B. schlosseri sequences. This may be preliminary evidence that some populations of B. schlosseri may actually be a different

species. We also found that the B. planus and B. violaceus sequences in GenBank were identical. This is an indication that some of the botryllid sequences in GenBank may have been generated from misidentified specimens. In order to clarify botryllid taxonomy, a comprehensive study that compares both morphological and genetic characteristics of samples from multiple geographic locations and of multiple botryllid species would be needed.

Analysis of the COI gene in B. schlosseri populations on Prince Edward Island revealed that there are two haplotypes of this species present on PEI. One of the haplotypes was found in every geographic location sampled on PEI while the other was only found in one colony growing on eelgrass in Savage Harbour. The presence of the second haplotype may be an indication that certain haplotypes of B. schlosseri grow preferentially on native substrates while others are more successful in settling on artificial substrate (where all previous sampling occurred). Alternatively, the second haplotype may be evidence that a new B. schlosseri haplotype was introduced to PEI between the 2008 and 2009 sampling seasons in a new invasion, while the predominant haplotype arrived in the initial invasion in 2001. Further sampling of B. schlosseri specimens on both artificial and native substrates in future could help elucidate this issue.

This low haplotype diversity in Prince Edward Island populations of B. schlosseri indicates that this species was likely transported to PEI in one initial invasion, and that the subsequent spread of this species to 12 other areas of PEI were due to local activity. Recreational boating, transfers of aquaculture species or gear and effluent from mussel processing plants may be local vectors which contributed to spread of this species across

PEI. The analysis of COI sequences from the B. schlosseri populations involved in this study indicate that the populations of this species on PEI are most closely related to populations in Yarmouth Nova Scotia, Magdalen Islands, Woods Hole and Salem, Massachusetts. This phylogenetic analysis suggests that the Massachusetts, Nova Scotia and Magdalen Island populations are possible source population for the PEI invasion. While there is no direct shipping traffic between Massachusetts and Prince Edward Island, there is a lot of shipping traffic between Massachusetts and Nova Scotia (DFO 2006). It is most likely that B. schlosseri was transported from Massachusetts to Nova Scotia via shipping traffic, and local vectors moved this tunicate from Nova Scotia to Prince Edward Island. The second haplotype of B. schlosseri detected on Prince Edward Island based on COI sequences grouped most closely with haplotype 3 from the Magdalen Islands, haplotype BR from Maine and haplotype HO from France. It is possible that the ferry that runs between Souris PEI and the Magdalen Islands is a vector for this species between the two regions. This would be a concern for both regions since Prince Edward Island currently has three invasive tunicate species that are not present in the Magdalen Islands, and an invasive tunicate Diplosoma listerianum was detected in the Magdalen Islands in 2008 but is not yet present on PEI.

The present findings highlight the impact that recreational boaters and local fisheries and aquaculture can have in spreading an invasive species once it has been brought to a new region. It is clear that educational programs at the local level are required to help minimize the local transfer of invasive species after they have been transferred to a new region. Once a new invasive tunicate is introduced to PEI waters, it has historically been difficult to contain in the initial region of the invasion. Therefore, minimizing the risks of the initial

invasion may be the best defense that this region has against future invasive tunicate species. This study also highlights the importance of regularly monitoring haplotypes in invasive populations to evaluate whether vectors continue to transport more organisms of an invasive species to the invaded region.

Phylogeographic studies of the three other invasive tunicate species in Prince Edward Island (B. violaceus, C. intestinalis and S. clava) should be conducted to evaluate the possible source populations of those invasions. This could help elucidate which vectors transported each invasive species to this region and help ensure that the same vector does not transport more invasive species to Prince Edward Island. In many Prince Edward Island bays, Ciona intestinalis has become the dominant fouling species despite the presence of other invasive tunicates in those bays (Ramsay et al. 2008). It would be particularly interesting to evaluate the genetic diversity of these C. intestinalis populations to determine if their competitive advantage may be linked to having a more genetically diverse population than the other invasive species, or whether one dominant haplotype has led to the success of this species in invading Prince Edward Island estuaries.

6.4 Conclusion

This study has illustrated that molecular biology techniques partnered with field based population monitoring can elucidate many factors surrounding the recent invasions of invasive tunicate in Prince Edward Island. Possible source populations and vectors for these invasions can be determined by phylogeographic analysis allowing for better management decisions and help in preventing future invasions from occurring by the same vectors. In addition, this work has shown that PCR and qRT-PCR based assays can be used

as efficient, sensitive, species specific and life stage specific tools that can also evaluate viability in invasive tunicates. As such, these assays would be powerful tools in monitoring and surveillance programs for invasive tunicate species in water samples.

6.5 References

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APPENDIX 1.Alignment of 18s rDNA sequences for *Botryllus schlosseri* (AB211066), *Botrylloides violaceus* (AY903927), *Styela clava* (L12442) and *Ciona intestinalis* (AB013017) with primer regions underlined or italicized.

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AB211066 ----------------------------GCTTGTCTCAAAGATTAAGCCATGCAGGTGTAA 33
AY903927 CCTGGTTGATCCTGCCAGTAGTGATATGCTTGTCTCAAAGATTAAGCCATGCAGGTGTAA 60
L12442   ------------------------------------------------------------
AB013017 ------------------TAGTGATATGCTTGTCTCAAAGATTAAGCCATGCAAGTCTAA 42

               BOTSCHLO18SF3
AB211066 GTACGAGTCGAACGAAAAGCGAAACTGCGAATGGCTCATTAAATCAGTCTTGGTTTATTT 93
AY903927 GTACGAGTTGA-CGTAAAGCGAAACTGCGAATGGCTCATTAAATCAGTCTTGGTTTATTT 119
L12442   ------------------------------------------------------------
AB013017 GTACGAGCTCT-CGTACAGCGAAACTGCGAATGGCTCATTAAATCAGTTATGGTTCATTT 101
              BOTSCHLO18SF1

AB211066 GGTCTCGAGAGCGAAGGTGGATAACTGTGGTAATTCCAGAGCTAATACATGCAATTAAGC 153
AY903927 GGTCTCGAGAGCGAAAGTGGATAACTGTGGCAATTCCAGAGCTAATACATGCAATTA-GC 178
L12442   ------------------------------------------------------------
AB013017 GATCGT-ATGGTTTACTTGGATAACTGTGGTAATTCTAGAGCTAATACATGCAAACA-GC 159
                    BOTSCHLO18SF4

AB211066 GCCGACTTCG-GGAGGCGCGCTTTTATTAGATCAAAA-CCGACCG--GCCTCGGCCGT-C 208
AY903927 GCCGACTTCG-GAAGGCGTGCTTTTATTGGATCAAAA-CCGACCG--GGTTCGCCCGT-C 233
L12442   ------------------------------------------------------------
AB013017 GCCGACTTCGCGGAGGCGTGCTTTTATCAGACCAAAAACCGACCGCGGCTCCGGCCGCGT 219

AB211066 TCTTTTGGTGACTCTGGATAACCACGCGGATCGCACGGTCTTGCGCCGGCGACGTACCAT 268
AY903927 TCTTTTGATGACTCTGGATAACCACGCGGATCGCGCGGTCTTGTGCCGGCGACAAACCAT 293
L12442   ------------------------------------------------------------
AB013017 TCGACTGGTGACTCTGGATAACCTGGCGGATCGCACGGTCTAGCACCGGCGACGGATCAT 279
                                            BOTVIOLET18SF1

AB211066 TCAAGTGTCTGACCTATCAACTTTCGAAGGTAAGCTACGGGCTTACCTTTGTGATAACGG 328
AY903927 TCAAGTGTCTGACCTATCAACTTTCGAAGGTAAGCTACGGGCTTACCTTTGTGATAACGG 353
L12442   ------------------------------------------------------------
AB013017 TCAAGTGTCTGCCCTATCAACTTTCGTCGGTACGGTATTCGCCTACCGA-GTTCTTACGG 338

AB211066 GTGACGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCA 388
AY903927 GTGACGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCA 413
L12442   ------------------------------------------------------------
AB013017 GTAACGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCA 398

AB211066 AGGAAGGCAGCAGGCGCGCAAATTACCCATTCCCGACACGGGGAGGTAGTGACGAAAAAT 448
AY903927 AGGAAGGCAGCAGGCGCGCAAATTACCCATTCCCGACACGGGGAGGTAGTGACGAAAAAT 473
L12442   ------------------------------------------------------------
AB013017 AGGAAGGCAGCAGGCGCGCAAATTACCCATTCCCGACGCGGGGAGGTAGTGACGAAAAAT 458
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AB211066 AACAATACAGGACTCTAACGAGGCCCTGTAATTGGAATGAGTACATTCTAAAACTCTTAA 508
AY903927 AACAATACAGGACTCTAACGAGGCCCTGTAATTGGAATGAGTACATTCTAAAACTCTTAA 533
L12442   ------------------------------------------------------------
AB013017 AACAATGCAGGACTCATTCGAGGCCCTGTAATTGGAATGAGTACACTTTAAAAGCTTTAA 518


AB211066 CGAGTATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAACAG 568
AY903927 CGAGTATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAACAG 593
L12442    ------------------------------------------------------------
AB013017 CGAGTATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAAAAG 578

                                                        BOTSCHLO18SR4
AB211066 TGTATGCTAAAGTTGTTGCGGTTGAAAAGCTCGTAGTTGGATCTTGGGCGGGTGCGGTCG 628
AY903927 TGTATGCTAAAGTTGTTGCGGTTGAAAAGCTCGTAGTTGGATATTGGGCGAGCGCGGTCG 653
L12442   --------AAAGTTGTTGCGGTTGAAAAGCTCGTAGTTGGATTTTGGGCGAGCGCCGCCG 52
AB013017 TATATATTTAAGTTGTTGCGGTTGAAAAGCTCGTAGTTGGATTTTGGGCGCGGGCGGTCG 638
                                                      BOTSCHLO18SR3

AB211066 GTCCGTCGCAGGGCGTG-CACTGGTCGCGCTCGCCTCGCCTTCGGTTCTCCGACGGTGCT 687
AY903927 GTCCGTCGCAGGGCGTG-CACTGGTCGCGTTCGCCTCGACTTCGGTTCTCCGTCGGTGCT 712
L12442   GTCCGTCGCAAGGCGTGTCACTGGTTGCGTTCGCCTCACCTTCGGTTCTCCGTCGGTGCT 112
AB013017 GTCCGTCGCGAGGCGTG-TACTGGTCGACCCGGCCTTACGTCCGGTTCTCCGGCGGTGCT 697
                 STYCLAV18SF1   CIONAINTEST18SF1

AB211066 CTTGACTGAGTGTCGCCGGTGGCCGAGAAGTTTACTTTGAAAAAATTAGAGTGTTCAAAG 747
AY903927 CTTGACTGAGTGTCGGCGGTGGCCGAGAAGTTTACTTTGAAAAAATTAGAGTGTTCAAAG 772
L12442   CTTGACTGAGTGTCGGCGGTGGCCGATAAGTTTACTTTGAAAAAATTAGAGTGTTCAAAG 172
AB013017 CTTGACTGAGTGTCGGCGGCGGCCGGAAAGTTTACTTTGAAAAAATTAGAGTGTTCAAAG 757


AB211066 CAGGCTGGTC-GCCTGAATAGTGTTGCATGGAATAATGGAATAGGACCTCGGTTCTATTT 806
AY903927 CAGGCTGGTC-GCCTGAATAGTGTTGCATGGAATAATGGAATAGGACCTCGGTTCTATTT 831
L12442   CAGGCTGTTC-GCCTGCATAGTGTTGCATGGAATAATGGAATAGGACCTCGGTTCTATTT 231
AB013017 CAGGCTCTCGAGCCTGAATAATGGTGCATGGAATAATGGAATAGGACCTCGGTTCTATTT 817


AB211066 TGTTGGTTTTCGGAACGTGAGGTAATGATTAAGAGGGACAGACGGGGGCGTCCGTACTCT 866
AY903927 TGTTGGTTTTCGGAACG-GAGGTAATGATTAAGAGGGACAGACGGGGGTGTCCGTACTCT 890
L12442   TGTTGGTTTTCGGAGCACGAGGTAATGATTAAGAGGGACAGACGGGGGCGTCCGTACTCT 291
AB013017 TGTTGGTTTTCGGAGCGCGAGGTAATGATTAAGAGGGACAGACGGGGGCATTCGTACTGT 877


AB211066 GCCGTTAGAGGTGAAATTCTTGGATCGGCGGAAGACGAACTACTGCGAAAGCATTCGCCA 926
AY903927 GCCGTTAGAGGTGAAATTCTTGGATCGGCGGAAGACGAACTACTGCGAAAGCATTCACCA 950
L12442   GCCGTTAGAGGTGAAATTCTTGGATCGGCGGAAGACGAACTACTGCGAAAGCATTCGCCA 351
AB013017 GCCGTTAGAGGTGAAATTCTTGGATCGGCGCAAGACGAACGACTGCGAAAGCATTTGCCA 937


AB211066 AGAATGTTTTCTTTAATCAAGAGCGAAAGTCAGAGGTTCGAAGACGATCAGATACCGTCC 986
AY903927 AGAATGTTTTCTTTAATCAAGAGCGAAAGTCAGAGGTTCGAAGACGATCAGATACCGTCC 1010
L12442   AGAATGTTTTCTTTAATCAAGAGCGAAAGTCAGAGGTTCGAAGACGATCAGATACCGTCC 411
AB013017 AGAATGTTTTCTTTAATCAAGAGCGAAAGTCAGAGGTTCGAAGACGATCAGATACCGTCC 997
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AB211066 TAGTTCTGACTATAAACGATGCCAACTAGCGATCGGGAGGCGTTACCATGACGACCTTCC 1046
AY903927 TAGTTCTGACTATAAACGATGCCAACTAGCGATCGGGAGGCGTTACCATGACGACCTTCC 1070
L12442   TAGTTCTGACTATAAACGATGCCAACTAGCGATCGGGAGGCGTTACCATGACGACCTTTC 471
AB013017 TAGTTCTGACTATAAACGATGCCAACTAGCGATCGGGGAGCGTTAATTTGACGACCTCCC 1057


AB211066 CGGCAGCTTCCGGGAAACCAAAGTCTTTGGGTTCCGGGGGAAGTATGGTTGCAAAGCTGA 1106
AY903927 CGGCAGCTTCCGGGAAACCAAAGTCTTTGGGTTCCGGGGGAAGTATGGTTGCAAAGCTGA 1130
L12442   CGGCAGCTTCCGGGAAACCAAAGTCTTTGGGTTCCGGGGGAAGTATGGTTGCAAAGCTGA 531
AB013017 CGGCAGCTTGCGGGAAACCAAAGTCTTTGGGTTCCGGGGGAAGTATGGTTGCAAAGCTGA 1117


AB211066 AACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACT 1166
AY903927 AACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACT 1190
L12442   AACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACT 591
AB013017 AACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACT 1177


AB211066 CAACACGGGGAAACTCACCCGGCCCGGACACAGGAAGGATTGACAGATTGAGAGCTCTTT 1226
AY903927 CAACACGGGGAAACTCACCCGGCCCGGACACAGGAAGGATTGACAGATTGAGAGCTCTTT 1250
L12442   CAACACGGGGAAACTCACCCGGCCCGGACACAGGTAGGATTGACAGATTGAGAGCTCTTT 651
AB013017 CAACACGGGAAATCTCACCCGGCCCGGACACAGTGAGGATTGACAGATTGAGAGCTCTTT 1237


AB211066 CTTGATTCTGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTT 1286
AY903927 CTTGATTCTGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTT 1310
L12442   CTTGATTCTGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTT 711
AB013017 CTTGATTCTGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTT 1297


AB211066 AATTCCGATAACGAACGAGACTCTGGCATGCTAAATAGTTACGCGACCTTCTCGGTCGGC 1346
AY903927 AATTCCGATAACGAACGAGACTCTGGCATGCTAAATAGTTACGCGACCTTCTCGGTCGGC 1370
L12442   AATTCCGATAACGAACGAGACTCTGGCATGCTAAATAGTTACGCGACCTTCTCGGTCGGC 771
AB013017 AATTCCGATAACGAACGAGACTCTGGCTTGCTAAATAGTTACGCGACCCTCCCGGTCGGC 1357


AB211066 GTCTAACTTCTTAGAGGGACTAGTGGCGTTTAGCCACACGAGATTGAGCAATAACAGGTC 1406
AY903927 GTTTAACTTCTTAGAGGGACTAGTGGCGTTTAGCCACACGAGATTGAGCAATAACAGGTC 1430
L12442   GTCTAACTTCTTAGAGGGACTAGTGGCGTTTAGCCACACGAGATTGAGCAATAACAGGTC 831
AB013017 GTTTAACTTCTTAGAGGGACTAGTGGCGTTTAGCCACACGAGATTGAGCAATAACAGGTC 1417


AB211066 TGTGATGCCCTTAGATGTCCGGGGCCGCACGCGCGCTACACTGAATGAATCAGCGTGT-- 1464
AY903927 TGTGATGCCCTTAGATGTTCGGGGCCGCACGCGCGCTACACTGAATGAATCAGCGAGT-- 1488
L12442   TGTGATGCCCTTAGATGTCCGGGGCCGCACGCGCGCTACACTGAGTGAAGCAGCGAGTGT 891
AB013017 TGTGATGCCCTTGAGTGTTCGGGGCCGCACGCGCGCTACACTGACCGGATCAGCGTGTCT 1477
                                                  STYCLAV18SR1

             BOTSCHOL18SR1
AB211066 CTCTCCTAGGCCGAAAGGTCCGGGTAACCCGTTGAACCTCATTCGTGATTGGGATAGGGA 1524
AY903927 CTAACCTGGGCCGAAAGGTCTGGGTAACCCGTTGAACCTCATTCGTGATTGGGATAGGGA 1548
L12442   CTAACCTAGGCCGAAAGGTCCGGGTAACCCGTTGAACCTCATTCGTGATTGGGATAGGGA 951
AB013017 CT-TCCTTGGCCGAAAGGTCCGGGAAACCCGTTGAACCCCCGGTGGTGATAGGGATAGGGA 1536
               BOTVIOLET18SR1                    CIONAINTEST18SR1
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AB211066 CTTGCAATTGTTTCCCTTGAACGAGGAATTCCCAGTAAGCGCAAGTCATCAGCTTGCGTT 1584
AY903927 CTTGCAATTGTTTCCCTTGAACGAGGAATTCCCAGTAAGCGCAAGTCATCAGCTTGCGTT 1608
L12442   CTTGCAATTGTTTCCCTTGAACGAGGAATTCCCAGTAA---------------------- 989
AB013017 ATTGCAATTATTTCCCTTGAACGAGGAATTCCCAGTAAGCGCGAGTCATTAGCTCGCGTT 1596


AB211066 GATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGTTTAGT 1644
AY903927 GATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGTTTAGT 1668
L12442   ------------------------------------------------------------
AB013017 GATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGTTTAGT 1656


AB211066 GAGATCCTTGGATCGGCCCCGTCGCGGCTGGCAACGGCCGCGTCGGCGCGTCGAGAAGAC 1704
AY903927 GAGATCCTTGGATCGGCCCCGTCGCGGCTGGCAACAGTCGAGTCGGCGCGTCGAGAAGAC 1728
L12442   ------------------------------------------------------------
AB013017 GAGGTCCTCCGATTGGACCCGGCGCGGTCGGCAACGTCCGCGCCGGCGT-CCGAAAAGAC 1715


AB211066 GATCAAACTTGATCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTT-------------- 1750
AY903927 GATCAAACTTGATCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCT 1788
L12442   ------------------------------------------------------------
AB013017 GATCTAACTTGATCATTTAGAGGAAGTAAAAGTCGTAACAA------------------- 1756


AB211066 ---------------
AY903927 GCAGATGGATCATTA 1803
L12442   ---------------
AB013017 ---------------
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APPENDIX 2. Alignment of COI sequences for *Botryllus schlosseri* (DQ340224) and *Ciona intestinalis* (AK116803) with primer regions underlined.

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DQ340224    ------------------------------------------------
AK116803    TGATTAACTCGATGAATATTTTCGACTAATCATAAAGATATTAGAACTTT 50

DQ340224    ------------------------------------------------
AK116803    GTATTTTATTTTCAGAACTTGGTCTGGAATTATTAGAACTTCTTTAAGTG 100

DQ340224    ----------------------------------GTGGTTAGAGACAGG 15
AK116803    TAATTATTCGTATAGAGTTATCTCAAAGAAGAAGAGTAATTAATAACAGA 150

DQ340224    CTATTGTATAATGTAATTGTAACTGCTCATGCCTTTGTGATGATCTTTTT 65
AK116803    CAAATTTATAATGTCGTTGTTACTTCTCATGCATTTGTTATAATTTTTTT 200
                        CIONAINTESTCOIF1

DQ340224    TTTTGTTATACCTATGATAATTAGGAGGTTTGGTAATTGGTTATTACCTT 115
AK116803    -TTTGTAATACCAATTATAATTAGAAGATTTAGAAATTGATTAATTCCTT 249
                              BOTSCHLOCOIF1

DQ340224    TAATAGTGGGGAGTCCAGATATGGCTTTTCCTCGATTAAATAATATAAGT 165
AK116803    TAATAGTAGGAGCTCCTGATATAGCTTTTCCTCGGATAAATAATATGAGT 299

DQ340224    TTTTGATTATTGCCCCCTGCTTTGTTTTTTCTTTTTAGAAGT---TCTAT 212
AK116803    TTTTGACTATTACCTCCTTCTTTTTTTTTATTATTAAAAAGTAGATTCTC 349
                        BOTSCHLOCOIF2

DQ340224    AATCGAGAGTGGAGTTAGGACCGGGTGAACTGTTTATCCTCCCCTTTCTA 262
AK116803    AGGTTCAGCTAGAGTAAGAACTGGGTGAACAGTTTACCCTCCTTTATCTG 399

DQ340224    GAAACTTAGCTCATTCTAGAGCTGCTTTGGATTGTGCTATTTTTT-CTTT 311
AK116803    CAAATATCAGACACTCTAATACTAGAGTTGATATAGCTATTTTTTTCTTT 449

DQ340224    ACATTTGGCTAGAGTGTCTAGTATTTTAAGATCTCTTAACTTTATGACTA 361
AK116803    ACATTTAGCTAGAGTTTCTAGTATTTTAAGATCAGTTAATTTTTTAGTTA 499

DQ340224    CTTTGTTTAATATAAAGGTAAAAGGTTGGGGACTTTTTTCTATATCTTTG 411
AK116803    CTTTATTTAATATAAAAAATAAAAGAAAATCAATAAGTAATTTAAGTTTA 549

DQ340224    TTTTGTTGAACTGTATTGGTCACTACTATCCTGTTATTATTATCTTTACC 461
AK116803    TTTTGTTGATCTTTAATTGTTACAACTATTTTACTAGTACTATCACTTCC 599
               BOTSCHLOCOIR1

DQ340224    TGTTTTGGCAGCTGCTATTACTATGCTGTTGTTTGATCGAAACTTCAATA 511
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AK116803    TGTTTTAGCTGCAGCAATTACTATATTATTATTTGATCGAAATTTTAATA 649


DQ340224    CTTCTTTTTTTGA------------------------------------ 524
AK116803    CTACGTTCTTTGATCCGAATGGGAGAAGAGATCCAATTTTATATCAACAT 699
            CIONAINTESTCOIR1
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